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PRINCIPAL INVESTIGATOR(S): Stephen Byers, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

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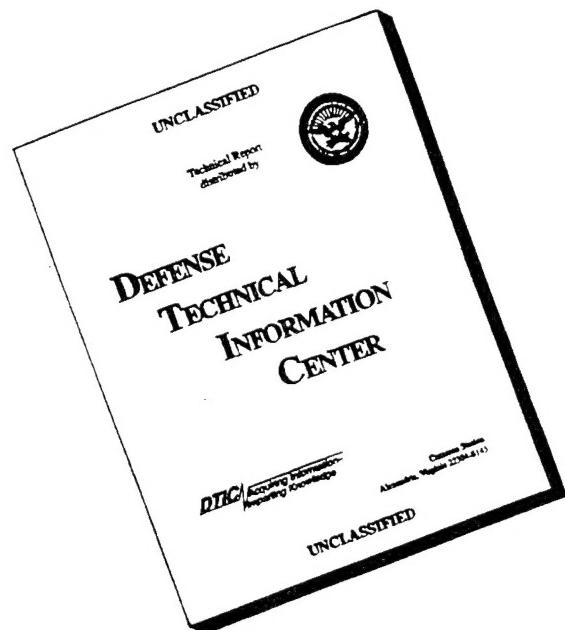
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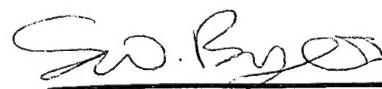
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INTRODUCTION

Nature of the Problem

Defects in cell-cell adhesion are commonly associated with tumor progression. There is evidence that alterations in the expression of the calcium-dependent cell adhesion molecule E-cadherin occur in a subset of invasive breast cancers and breast cancer cell lines. However, many invasive breast cancers and metastases are E-cadherin positive. Preliminary results indicate that breast tumor progression may more often be accompanied by alterations in the expression and function of several cadherin-associated molecules that are essential for cadherin-mediated cell-cell adhesion. It is the aim of this proposal to test the hypothesis that, in addition to the occasional loss of E-cadherin expression, breast tumor progression is more realistically modeled by a defect in cell-cell adhesion resulting from an alteration in any one or more of the steps (molecules) required for E-cadherin function. We will take two fundamental approaches. Firstly, we will use two methods for "non-specifically" assessing E-cadherin function and cell-cell adhesive strength in breast tumor samples and cell lines. Secondly, we will specifically investigate the molecular mechanisms that lead to defects in cell-cell adhesion by examining (and manipulating) the expression and phosphorylation state of several E-cadherin associated molecules in breast tumors and cell lines.

Background and Previous Work

Cell Adhesion, Cell junctions and Cancer The concept that alterations in cell to cell adhesion and intercellular communication are involved in tumorigenesis and tumor progression is certainly not new (Loewenstein, 1979). However, it is only recently that the molecular basis underlying these changes has begun to be addressed. Homotypic cell-cell adhesion molecules (CAMs) and cell-cell junction molecules have now been implicated as tumor and metastasis suppressor genes in several systems (Shiozaki et al. 1991; Shimoyama et al. 1989; Schipper et al. 1991; Mareel et al. 1991; Frixen et al. 1991; Mahoney et al. 1991; Sommers et al. 1992). A gene often deleted in colon carcinoma (DCC) and associated with colon tumor progression is likely to be a homotypic CAM of the immunoglobulin superfamily (Fearon and Vogelstein, 1990). Of most interest to the present proposal are the calcium-dependent class of CAMs, cadherins, characteristically expressed by cells of epithelial origin (Takeichi, 1990; Takeichi, 1991). Cadherin-mediated adhesion is fundamentally involved in the organization of epithelial tissues during development, and manipulations of cadherin function result in profound disturbances of tissue organization (Takeichi, 1990; Takeichi, 1991; Hirai et al. 1989; Gallin et al. 1986). Several drosophila tumor suppressor genes are either cadherins or junction associated molecules and reduction in cadherin expression has been associated with the malignant phenotype in many advanced human carcinomas (Shiozaki et al. 1991; Shimoyama et al. 1989; Schipper et al. 1991; Mareel et al. 1991; Frixen et al. 1991; Sommers et al. 1992; Mahoney et al. 1991). In order for cadherins to function in cell-cell adhesion and promote the formation of junctions several other associated molecules need to be expressed (Herrenknecht et al. 1991; Nagafuchi et al. 1991; Takeichi, 1991; Hirano et al. 1992). These molecules known as catenins, link the cadherins to the underlying actin cytoskeleton and are probably involved in propagating adhesion-related signalling (Hirano et al. 1992; Matsuyoshi et al. 1992). Our preliminary results show that the expression of certain catenins is lost in malignant breast carcinoma cells (Hirano et al. 1992). Other studies have indicated that the phosphorylation state of catenins can also influence the transformed phenotype (Matsuyoshi et al. 1992). The mechanism whereby alterations in cadherin-mediated adhesion

affects cell proliferation, morphological differentiation and invasion is unknown. All differentiated epithelial cell collectives are linked by gap junctions permeable to intracellular calcium. In this situation, changes in intracellular calcium can be propagated rapidly among communicating cells. It is therefore not surprising that many tumor cells including breast tumor cells are deficient in gap junctional communication ((Lee et al. 1992) and references therein). Interestingly, transfection of E-cadherin into squamous cell carcinoma cells lacking gap junction function results in the expression of the gap junction proteins connexins and the assembly of functional gap junctions (Jongen et al. 1991).

E-cadherin in Breast Cancer The presence of lymph node and distant metastases predict poor prognosis for cancer patients. For example, in a large clinical trial, percent treatment failure at 5 years for patients with no lymph node involvement upon histological examination was 13%, for those with 1-3 positive nodes the failure rate was 39% and for those with >4 positive nodes the failure rate was 69% (reviewed in (Yeatman and Bland, 1991)). These results underscore the necessity for research designed to understand the process of metastasis and to discover molecular markers that will predict whether a given tumor is likely to metastasize. The study of E-cadherin and associated protein expression and function in breast cancer cells can potentially provide information pertinent to both of these aims.

Several studies have examined the expression of E-cadherin in human breast cancer tissues (Gamallo et al. 1993; Oka et al. 1993; Rasbridge et al. 1993). Loss or reduction of E-cadherin immunostaining was observed in a proportion of samples in each study. Normal breast epithelial structures consistently stain at cell-cell borders for E-cadherin. In one study (Oka et al. 1993), 53% of 120 tumors had reduced E-cadherin expression (defined as >10% of cells being E-cadherin-negative). The majority of the samples examined in this study were invasive ductal carcinomas, the most common form of breast cancer diagnosed. Loss of E-cadherin expression correlated with poorer differentiation state and with higher stage (T, N and M). In particular, 86% of samples from patients with distant metastasis (M_1) had reduced E-cadherin staining whereas 47% of samples from patients with no known distant metastasis (M_0) had reduced E-cadherin expression (Oka et al. 1993). Similar results were reported in a smaller study by Gamallo et al. (Gamallo et al. 1993). A third study examined a larger number of invasive lobular carcinomas (Rasbridge et al. 1993). Complete loss of E-cadherin expression was detected in 29 of 35 samples. The remaining 6 samples had a diffuse staining pattern for E-cadherin. The ductal carcinoma samples had variable intensities of staining for E-cadherin although the proportions of E-cadherin-negative cells in each sample was not quantitated. Taken together, these results indicate that E-cadherin expression is lost in a significant proportion of lobular carcinoma specimens and reduced E-cadherin expression correlated with higher stage (poorer prognosis) and poorer differentiation in invasive ductal carcinomas.

One difficulty in comparing the results of E-cadherin staining of tumor tissues is the inconsistency between observers regarding when to classify a tumor as "E-cadherin-positive" or "E-cadherin-negative". In the study by Oka et al. (Oka et al. 1993), tumors with >10% of cells displaying no E-cadherin immunostaining were classified as having "reduced expression". However, in many studies no such cutoff was used (e.g. (Gamallo et al. 1993)). Many studies also describe "diffuse" or "disorganized" or "reduced" staining patterns for E-cadherin (Gamallo et al. 1993; Rasbridge et al. 1993; Eidelman et al. 1989). These descriptions may indicate a defect in connection of E-cadherin to the actin cytoskeleton in these samples which could lead to a loss of adhesive function even in the presence of immunoreactive E-cadherin.

Although a trend between increasing stage and reduced E-cadherin expression was observed in breast cancer (Oka et al. 1993), the ability to predict metastatic spread based on expression of E-cadherin in a primary breast tumor is uncertain. An analysis of E-cadherin expression in 19 lymph node metastases and in their primary tumors was performed (Oka et al. 1993). Of the primary tumors, six were E-cadherin-positive, five were E-cadherin-negative and eight had a mixed phenotype. Five of the six lymph node metastases from E-cadherin-positive primary tumors were E-cadherin-positive and one was mixed. All five of the lymph node metastases from E-cadherin-negative primary tumors were E-cadherin-negative. Among the lymph node metastases from the eight mixed primary tumors, three were E-cadherin-positive, two were E-cadherin-negative and two were mixed. The fact that five of the eight lymph node metastases from mixed primary tumors contained E-cadherin-positive indicates that a selection for E-cadherin-negative cells in the metastatic process does not occur.

Rationale and Hypothesis to be Tested The discussion above together with our preliminary data has led us to conclude that E-cadherin expression alone is a poor predictor of breast tumor invasive potential and metastatic spread. Significant loss of heterozygosity (LOH) of chromosomal locus 16q occurs in several carcinomas including breast (Lindblom et al. 1993; Nishida et al. 1992; Carter et al. 1990). The human E-cadherin gene is localized to 16q22.1 and it is possible that an apparent reduction in staining intensity in some tumors may be due to this LOH. In breast cancer 16q LOH is correlated with distant metastatic spread. Although loss of functional homotypic cell-cell adhesion and intercellular communication are clearly associated with the transformed malignant epithelial cell phenotype this is not necessarily due to loss of cadherin expression or LOH at the cadherin locus. A defect in any one of the molecules involved in cadherin function, or a change in any of the pathways involved in cadherin responsive intra- and inter-cellular signalling could also result in the same phenotype. Other cell-cell adhesion molecules not discussed here are also likely to be affected. In other words, the two important carcinoma cell adhesion related phenotypes of 1) alterations in contact dependent growth and 2) invasion and metastasis, can be achieved in many different ways. Based on our own preliminary results and those in the literature we have calculated that there are several hundred potential routes whereby functional cell-cell adhesion could be altered during carcinogenesis and result in these phenotypes. Bearing in mind that we are limited by current knowledge this number is likely to be conservative. Not surprisingly, more than a dozen lesions in the cadherin-related adhesion system alone have already been uncovered in various carcinomas and cell lines (see discussion above). Whilst it is clearly of great importance to continue cataloging these molecular changes, indeed we propose to do so in one of our specific aims, it is equally important to develop methods in which functional cell-cell adhesion can be assessed directly. Such methods should uncover any defect in cell-cell adhesive strength no matter what the underlying molecular basis.

METHODS AND RESULTS

Task 1. To test the hypothesis that cell-cell adhesive strength and E-cadherin triton solubility is correlated with functional E-cadherin-mediated cell-cell adhesion. Years 1-4
The first "non-specific" approach that we will use for assessing tumor cell-cell adhesion strength is based on our recently described laminar flow assay (Byers et al. 1995). Using this assay developed originally to investigate cell-substratum interactions, we hypothesized that we should be able to distinguish cell-cell adhesive strength among tumor cells which express functional and non-functional E-cadherin. We will extend these studies to many more cell lines and to tumors derived

from these lines. We will refine our procedures using tumors derived from cells which we already know express functional and non-functional E-cadherin and which are of known adhesive strength *in vitro*. It is not the goal of this specific aim to use these direct assays of cell-cell adhesive strength as a routine screening procedure for breast cancer. For each sample we will correlate cell-cell adhesion strength with E-cadherin triton solubility (see below). We will restrict our analyses to tumors in which E-cadherin is present (but perhaps non-functional). In this way we can be certain that the cell aggregates that we analyze are derived from the tumor itself rather than any stromal elements which may contaminate it, since these will be E-cadherin negative and will not exhibit calcium-dependent cell-cell adhesion. We are not so interested in E-cadherin negative tumors since these have an obviously demonstrable lesion in cell-cell adhesion.

Task 1. Methods and Results (refer to figures in appendix 1)

Preparation of cells and assays of cell-cell adhesion strength Cells growing in culture are trypsinized and resuspended at a concentration of 2×10^6 cells/ml. Finely minced tumor specimens (100-500mg) will be incubated at 37°C in trypsin/collagenase with regular trituration for 30 min-2h. Single cells and small aggregates will be isolated by centrifugation after the removal of undigested tissue on a nylon mesh. 5 ml of the cell suspension (2×10^6 cells/ml) are placed in a T-75 tissue culture flask and gassed with 5% CO₂. Flasks are then rotated at 140 rpm at 37°C for 4 h to allow aggregates to form. At the end of this time the cell aggregates are collected and used for laminar flow assays as described below.

A parallel flow chamber of uniform width was used in the laminar flow assays (Chien and Sung, 1987). The chamber consists of (a) an upper plate having appropriate openings for the delivery of the fluid into and out of the channel, (b) a gasket with an opening in the form of a channel, (c) a transparent bottom plate (Grade #1 coverslip) and (d) top and bottom stainless steel cover plates with observation slots. The bottom plate, the gasket, and the base plate are fastened between the cover plates. The entry port of the chamber is connected through a valve and teflon tubing to two syringes, one filled with cell suspension and the other filled with suspending medium. Before use in the flow chamber glass coverslips were coated with laminin (10 µg/cm²) or collagen type I (10µg/cm²) as described earlier (Tozeren et al., 1994).

A syringe pump (Harvard Apparatus) was used to pump medium into the chamber at specified flow rates. The shear stress on the bottom plate of the chamber along the direction of flow, τ (dyn/cm²), was evaluated using the following equation, assuming Poiseuille flow:

$$\tau = 6\mu Q / h^2 w \quad (1)$$

where μ (0.01 dyn-s/cm²) is the viscosity of the medium, Q (cm³/s) is the flow rate, h is the gap thickness of the channel (0.012 cm) and w (1 cm) is the width of the chamber (Chien and Sung, 1987).

Laminar flow assays were initiated by placing the flow chamber on the stage of an inverted microscope (Diaphot, Nikon Inc., Garden City, NJ) equipped with 10X and 40 X Hoffman and brightfield objective lenses. The cell suspension was gently infused into the flow channel and cell aggregates allowed to interact with the matrix protein-coated glass coverslip for 20 minutes under static conditions. Flow was then initiated at $\tau = 1.75$ dyn/cm² and the flow rate increased at 30 second or one minute intervals up to a maximum value of $\tau = 100$ dyn/cm².

A video camera (DAGE-MTI) was attached to the side port of the microscope to record the deformation / disaggregation response of cell aggregates to imposed laminar flow. The times were displayed on the video monitor with a data mixer (Vista Electronics, La Mesa, CA) and the length and width of the cell aggregates before and during flow were determined using a position analyzer mixer (Vista Electronics, La Mesa, CA) that provided a digital readout proportional to the distance between two sets of vertical and horizontal lines.

Flow-induced disaggregation of both small (2-6) cells and large aggregates were recorded. Large aggregates were defined as those whose largest dimension before the imposition of flow was 70-140 μm . Large carcinoma cell aggregates typically contained multiple layers of cells with many cells adherent to neighboring cells but not to the planar substratum.

A detachment event was said to occur when a single cell or a small cell aggregate detached from the parent aggregate in response to the imposed flow. In each experiment with a large aggregate, the number of detachment events during a one minute interval of infusion at a constant level of shear stress was determined. The total number of disaggregation events performed with each cell type varied between 8 and 14. The mean and standard deviation of the number of detachment events were computed as a function of the fluid shear stress imposed on the laminin or collagen-coated glass coverslip. The mean value was denoted as the frequency of disaggregation.

To investigate E-cadherin detergent solubility in breast tumor samples and breast cancer cell lines. As stated above, we hypothesize that E-cadherin that is fully functional in cell-cell adhesion will be present as a triton-insoluble pool at points of cell-cell contact. Our preliminary results and those of others indicate that defects in the linkage of E-cadherin to the cytoskeleton result in the loss of this triton-insoluble pool. Therefore, assays which can directly assess E-cadherin detergent solubility will help determine the ability of E-cadherin to mediate strong cell-cell adhesion. There are two ways in which triton-solubility can be determined. Firstly, a biochemical approach in which cell or tissue extracts are partitioned into detergent soluble and insoluble phases. Secondly, an immunocytochemical approach in which cells or tissue sections are treated with triton prior to fixation. Our preliminary results indicate that the biochemical approach does not yield interpretable results in detergent extracted cell lines. However, immunocytochemistry of cells with and without triton treatment reliably predicts the cell-cell adhesion strength. In other words, cells in which a triton-insoluble pool of E-cadherin exists at points of cell-cell contact exhibit strong cell-cell adhesion whereas cells in which no such pool exists do not. The forces required for disaggregation of the two categories differ by at least one order of magnitude.

Immunofluorescence microscopy of triton-insoluble E-cadherin Immunofluorescence microscopy of Triton X-100-insoluble E-cadherin is performed based on the protocol of Shore and Nelson (Shore and Nelson, 1991). Frozen sections of breast tumor material or cells grown on glass coverslips or 8-well chamber slides precoated with 50 $\mu\text{g}/\text{ml}$ collagen I to enhance attachment during Triton X-100 treatment are washed once with PBS and incubated for 10 minutes at room temperature in CSK buffer (Shore and Nelson, 1991) with or without 0.5% Triton X-100 (Sigma). [CSK buffer - 10 mM PIPES, pH 6.8, 50 mM NaCl, 3 mM MgCl₂, 0.5% Triton X-100, 300 mM sucrose, 0.1 mg/ml RNase, 0.1 mg/ml DNase]. Cells are then fixed in 1.75% formaldehyde in PBS for 10 minutes at room temperature. We will also test the effectiveness of including triton in the fixative. Following blocking with 3% ovalbumin (Sigma) in PBS, samples are incubated with the primary antibodies described below diluted in PBS with 5% normal goat serum at 37°C for one hour. FITC-conjugated secondary antibodies (Cappell, West

Chester, PA) are used at 1:2500 dilution. Samples will be visualized by epi-illumination on a Nikon Diaphot photomicroscope linked to an MRC-600 Laser Scanning Confocal Microscope (LSCM).

The Role of E-cadherin in the Response of Tumor Cell Aggregates to Lymphatic, Venous and Arterial Flow: Measurement of Cell-Cell Adhesion Strength. During the first 12 months of the grant we have further refined our biophysical methods for measuring cell-cell adhesion strength and have confirmed that the presence of detergent-insoluble E-cadherin or β -catenin correlates with strong cell cell adhesion. Some of this work was recently published (appendix 1) and is summarized below.

Defects in the expression or function of the calcium dependent cell-cell adhesion molecule E-cadherin are common in invasive, metastatic carcinomas. In the present study the response of aggregates of breast epithelial cells and breast and colon carcinoma cells to forces imposed by laminar flow in a parallel plate flow channel was examined. Although E-cadherin negative tumor cells formed cell aggregates in the presence of calcium, these were significantly more likely than E-cadherin positive cell aggregates to disaggregate in response to low shear forces, such as those found in a lymphatic vessel or venule ($<3.5 \text{ dyn/cm}^2$). E-cadherin positive normal breast epithelial cells and E-cadherin positive breast tumor cell aggregates could not be disaggregated when exposed to shear forces in excess of those found in arteries ($> 100 \text{ dyn/cm}^2$). E-cadherin negative cancer cells which had been transfected with E-cadherin exhibited large increases in adhesion strength only if the expressed protein was appropriately linked to the cytoskeleton. These results show that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. Since a primary route of dissemination of many carcinoma cells is to the local lymph nodes these results point to a novel mechanism whereby defects in cell-cell adhesion could lead to carcinoma cell dissemination.

Task 2. To measure the expression and phosphorylation state of cadherin-associated proteins in breast tumors and cell lines (Years 1-4). We will examine the expression and phosphorylation state of the cadherin-associated proteins alpha catenin, beta catenin and plakoglobin in breast tumors and cell lines.

Task 2. Methods and Results (refer to figures in appendix 2)

Immunoprecipitation and Western blot analysis Immunoprecipitations are performed based on the method of Shore and Nelson (Shore and Nelson, 1991). Frozen tumor samples (1-5 mg) are homogenized and partitioned into triton soluble and insoluble fractions as described by Shore and Nelson (Shore and Nelson, 1991) with the exception that 2mM H₂O₂ and 1 mM sodium orthovanadate are included in the lysis buffer. Cells are plated at a density of 1x10⁶ cells/well of a 6-well plate (Costar) in IMEM supplemented with 5% heat-inactivated fetal bovine serum. When super-confluent, cells are metabolically labelled with ³⁵S-methionine and ³⁵S-cysteine. Media is changed to IMEM without methionine and cysteine with HL-1 (Ventre Laboratories, Inc.) for 30 minutes. Then cells are incubated for four hours in IMEM without methionine and cysteine with HL-1 containing 0.25 mCi/ml TRAN³⁵S-LABEL (ICN). Cells are treated for 15 minutes with 2 mM H₂O₂ and 1 mM sodium orthovanadate, harvested and partitioned into Triton X-100 soluble and insoluble fractions. Extracts, e.g. 0.5 ml corresponding to half of the extract from one well or

equivalent to 0.1-0.5 mg of tumor tissue, are preincubated with 10 μ g mouse IgG and 10 μ l Protein G-Sepharose 4 Fast Flow (Pharmacia LKB) for one hour rocking at 4°C. The supernatant is incubated for one hour on ice with 1 ug HECD IgG. 10 μ l Protein G are added and incubation continued 60 to 90 minutes further at 4°C with rocking. The precipitates are washed in HSB, centrifuged through 33% sucrose in HSB, washed in HSB with 1M NaCl, and washed in LSB (Shore and Nelson, 1991). All solutions contain 1 μ g/ml aprotinin and 1 μ g/ml leupeptin and for the analysis of phosphotyrosine levels, 2mM H₂O₂ and 1 mM sodium orthovanadate. Samples are elecrophoresed on 10% SDS-PAGE gels. Western blots are performed using anti-phosphotyrosine and are visualized using alkaline phosphatase-conjugated secondary antibodies (Promega, Madison, WI) as described previously (Sommers et al. 1992). For metabolically labelled cell lines equivalent loading can be ascertained simply by exposing the blot to XAR film. For tumor extracts we will probe parallel blots with HECD to confirm that similar amounts of immunoprecipitated E-cadherin were loaded. This is an important control since some of the more advanced tumor samples may have reduced E-cadherin expression.

β -Catenin Associates With A Kinase And Is Phosphorylated On Serine And Threonine Residues. During the course of the first 12 months of the grant we carried out experiments to investigate the nature of catenin and cadherin phosphorylation in breast cancer cells. Our preliminary results showed that β -catenin is constitutively tyrosine phosphorylated in some breast cancer cells, however recent experiments demonstrated additional serine and threonine phosphorylation. In fact serine and threonine residues rather than tyrosine residues are the major target for kinases in this system. These results are summarized below.

β -catenin is an E-cadherin-associated molecule essential for the proper regulation of cell-cell adhesion. In Drosophila, the β -catenin homolog *armadillo*, an essential component of the wingless signaling pathway, is regulated by the activity of *zeste-white-3*, a homologue of glycogen synthase kinase-3 which is a mammalian serine/threonine kinase involved in many signaling pathways. Recent data from this laboratory indicates that increased β -catenin expression following treatment of breast cancer cells with retinoic acid (RA) may mediate increases in cell-cell adhesion strength. Inhibition of serine phosphatase activity increased the adhesive phenotype whereas inhibition of serine kinase activity completely reversed the morphological changes associated with RA treatment. In contrast, altered tyrosine phosphorylation of β -catenin is associated with increased invasiveness and decreased cell-cell adhesion strength in certain cell lines. In the present study immunoprecipitation and *in vitro* kinase analyses of detergent extracts from E-cadherin-negative invasive breast cancer cells showed that β -catenin associates with a kinase. Phosphoaminoacid (PAA) analyses showed that serine/threonine phosphorylation of β -catenin by the associated kinase was many fold higher than tyrosine phosphorylation. Cell lines transfected with E-cadherin and with E-cadherin lacking the β -catenin binding region were used to demonstrate that β -catenin is associated with, and can be phosphorylated by this kinase in complexes both with and without E-cadherin. Immunoprecipitated β -catenin is also phosphorylated in detergent extracts from metabolically labeled cells.

Task 3. Statistical analyses (years 3-4). Results will be correlated with tumor stage, blood vessel count, lymph node status, the expression of prognostic markers and period of metastasis-free survival. We are presently accumulating data on phosphorylation status and detergent solubility of cadherins and catenins in breast tumor tissues. This will continue until year 3 when we should have enough material to carry out a statistical analysis

Task 4. To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength (Years 1-4). We will examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength by directly examining the effects of kinase inhibitors and plakoglobin transfection on cell-cell adhesion strength using biophysical methods.

Task 4. Methods and Results (refer to figures in appendix 3)

To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion. Preliminary results showed that in two invasive cell lines β -catenin is constitutively heavily tyrosine phosphorylated. In this specific aim we will test the hypothesis that this hyperphosphorylation is the cause of the failure of transfected E-cadherin to alter the phenotype of the cells. In pilot experiments we have found that only one of the cell lines (BT549) responds to tyrosine kinase inhibitors by alterations in E-cadherin-mediated adhesion. We have used several classes of kinase inhibitors the most effective being herbimycin A. We are continuing these experiments and will ascertain the response of the cells in terms of adhesion strength, restriction of E-cadherin to cell-cell contact sites, and triton solubility. The second cell line (HS578T) does not respond to any of the tyrosine kinase inhibitors even at very high doses. Unlike BT549 cells which express low but detectable levels of the cadherin-associated molecule plakoglobin, HS578T cells do not express plakoglobin. We will determine whether the lack of plakoglobin is responsible for the failure of HS578T cells to respond to E-cadherin transfection by expressing plakoglobin cDNA in the cells.

The effects of kinase inhibitors on E-cadherin localization and triton solubility, cell-cell adhesion strength and β -catenin phosphorylation. The effect of herbimycin (100 ng/ml) on E-cadherin distribution has already been tested in E-cadherin transfected BT549 cells. After an overnight incubation in herbimycin many cells take on a more epithelial phenotype and E-cadherin becomes concentrated at points of cell-cell contact. We will continue these experiments and analyze the triton solubility of E-cadherin and the phosphorylation state of β -catenin as described above. In order to test alterations in adhesive strength we will allow the cells to aggregate and carry out laminar flow assays.

The effects of plakoglobin transfection on cell-cell adhesion strength. Plakoglobin negative E-cadherin transfected HS578T cells have been transfected with plakoglobin using the methodology we described for E-cadherin and vimentin transfections (Sommers et al. 1992; Sommers et al. 1993). Drs. Cowin and Franke have provided us with plakoglobin cDNA hPgca2.1 cloned into the ECOR1 site of Pl64J (Franke et al. 1989). We subcloned this into a CMV driven expression vector and transfected E-cadherin expressing HS578T cells as we described previously (Sommers et al. 1992; Sommers et al. 1993). Since these cells have already been selected for hygromycin resistance we co-transfected with a neomycin-resistance plasmid. Plakoglobin expressing clones have been selected by immunocytochemical staining. During the next few months selected clones will be characterized with respect to E-cadherin localization and triton solubility, responsiveness to kinase inhibitors, and cell-cell adhesion strength. If marked alterations in these characteristics occur as a result of plakoglobin transfection we will carry out *in vitro* invasion studies as we described earlier (Sommers et al. 1991).

Retinoids Increase Cell-Cell Adhesion Strength, β -Catenin Protein Stability and Localization to the Cell Membrane in a Breast Cancer Cell Line. A Role for Serine Kinase

Activity. During the present funding period we found that retinoic acid treatment of certain breast cancer cells dramatically increased cell-cell adhesion strength. Although these studies were not proposed in the original proposal the results are included here since they are directly relevant to the overall goal of the proposal. The expansion of the project to include the action of chemopreventive agents such as retinoids on the adhesive phenotype of breast cancer cells pushes the work into a more translational, treatment oriented direction. In this study we showed that a breast cancer cell line (SKBR3) which expresses no E-cadherin very low levels of β -catenin protein and exhibits a poorly adhesive phenotype in Matrigel, responds to retinoic acid (RA) by a marked increase in epithelial differentiation. Specifically, treatment of cells with all trans RA, 9-cis RA or a RA receptor α -specific ligand resulted in a large increase in cell-cell adhesive strength and stimulated the formation of fused cell aggregates in Matrigel. A retinoid X receptor-specific ligand was ineffective. Exposure of cells to RA for as little as 4 h was sufficient to maintain the adhesive phenotype for at least 4 days. The effects of RA required protein and RNA synthesis but were not mediated by factors secreted by stimulated cells, nor by direct cell contact and did not require serum. These RA-induced morphological effects were completely reversed by growing cells in 50 μ M Ca⁺⁺ suggesting a mechanism involving a RA-induced increase in Ca⁺⁺-dependent adhesion. Consistent with this, β -catenin protein levels were markedly elevated in the RA-treated cells and β -catenin became localized to a Triton-insoluble pool at regions of cell-cell contact. No change could be detected in β -catenin steady state mRNA levels but RA did increase β -catenin protein stability. Treatment of cells with low calcium medium did not prevent the RA-induced increase in total β -catenin protein but did prevent its movement to a Triton-insoluble pool at the cell membrane. Among several kinase inhibitors, only the broad spectrum kinase inhibitor staurosporine and the protein kinase C inhibitor bisindolylmaleimide reversed the morphological changes induced by RA. Like treatment with low calcium medium, these inhibitors did not prevent the RA-induced increase in total β -catenin protein levels but completely prevented the movement of β -catenin to the cell membrane. These results, point to a role for β -catenin and serine kinase activity in mediating the action of RA in epithelial differentiation.

CONCLUSIONS

Implication of the Completed Research

The major conclusions of the first year of work are:

- 1) Biophysical techniques can be used to accurately measure cell-cell adhesion strength. Results to date show that high cell-cell adhesion strength is correlated with the presence of detergent-insoluble E-cadherin. Our results show that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. Since a primary route of dissemination of many carcinoma cells is to the local lymph nodes these results point to a novel mechanism whereby defects in cell-cell adhesion could lead to carcinoma cell dissemination.
- 2) Investigations of the phosphorylation state of β -catenin have given us some unexpected results. Serine and threonine phosphorylation rather than tyrosine phosphorylation seems to be the major factor in regulating β -catenin function in cell-cell adhesion. Our future experiments will be modified accordingly to take this into account.

- 3)** A well known chemopreventive agent, retinoic acid, acts on certain breast cancer cells to increase cell-cell adhesion strength and leads to a decrease in the detergent solubility of β -catenin. This expands the project into a more translational area.

Recommended Changes

- 1)** We will expand our studies of phosphorylation to include serine and threonine residues as well as tyrosine residues.
- 2)** We will investigate in more detail the mechanism whereby retinoic acid increases cell-cell adhesion strength.

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Role of E-cadherin in the response of tumor cell aggregates to lymphatic, venous and arterial flow: measurement of cell-cell adhesion strength

Stephen W. Byers^{1,*}, Connie L. Sommers¹, Becky Hoxter¹, Arthur M. Mercurio² and Aydin Tozeren³

¹Department of Cell Biology and the Lombardi Cancer Center, Georgetown University Medical Center, Washington DC 20007, USA

²Laboratory of Cancer Biology, Deaconess Hospital, Harvard Medical School, Boston MA 02115, USA

³Department of Mechanical Engineering, The Catholic University of America, Washington DC 20064, USA

*Author for correspondence

SUMMARY

Defects in the expression or function of the calcium dependent cell-cell adhesion molecule E-cadherin are common in invasive, metastatic carcinomas. In the present study the response of aggregates of breast epithelial cells and breast and colon carcinoma cells to forces imposed by laminar flow in a parallel plate flow channel was examined. Although E-cadherin negative tumor cells formed cell aggregates in the presence of calcium, these were significantly more likely than E-cadherin positive cell aggregates to disaggregate in response to low shear forces, such as those found in a lymphatic vessel or venule ($<3.5 \text{ dyn/cm}^2$). E-cadherin positive normal breast epithelial cells and E-cadherin positive breast tumor cell aggregates could not be disaggregated when exposed to shear forces in excess of those found in arteries ($>100 \text{ dyn/cm}^2$). E-cadherin

negative cancer cells which had been transfected with E-cadherin exhibited large increases in adhesion strength only if the expressed protein was appropriately linked to the cytoskeleton. These results show that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. Since a primary route of dissemination of many carcinoma cells is to the local lymph nodes these results point to a novel mechanism whereby defects in cell-cell adhesion could lead to carcinoma cell dissemination

Key words: cadherin, tumor, adhesion

INTRODUCTION

Alterations in cell-cell and cell-extracellular matrix adhesion properties are consistently associated with the progression of carcinoma from a non-invasive to an invasive, metastatic phenotype (Liotta and Stetler Stevenson, 1991; Liotta, 1992; Takeichi, 1991; Albelda and Buck, 1990; Hynes, 1992). Several families of adhesion molecules have been implicated in these changes including the cadherins and integrins (Schipper et al., 1991; Frixen et al., 1991; Hynes, 1992). The expression of the calcium-dependent cell-cell adhesion molecule E-cadherin is reduced or completely lost in some invasive carcinomas and carcinoma cell lines (Schipper et al., 1991; Shimoyama et al., 1989; Shiozaki et al., 1991). Although these cells are likely to be capable of forming aggregates using alternative adhesion pathways, the loss of E-cadherin expression and/or function is generally thought to aid the local invasion process (Frixen et al., 1991; Behrens et al., 1989). Nevertheless, in other studies we found that most E-cadherin negative breast cancer cell lines were not more invasive than E-cadherin positive cells (Sommers et al., 1991). Instead, the highly invasive phenotype was invariably associated with expression of the mesenchymal intermediate filament protein

vimentin. Disruption of E-cadherin function in E-cadherin positive breast cancer cells resulted in the loss of cell-cell contact but did not result in the cells becoming more invasive (Sommers et al., 1991). Similarly, transfection of E-cadherin into invasive vimentin positive cells did not reverse the invasive phenotype even though it allowed the transfected cells to aggregate specifically with E-cadherin transfected fibroblasts (Sommers et al., 1994). Clearly, in this system loss of functional E-cadherin expression does not necessarily lead to an invasive phenotype. Although it is possible, in certain circumstances, that complete cell-cell detachment might be a required step in local invasion, in many developmental and clinical examples and in other instances of tissue remodeling, invasion of a surrounding tissue or matrix is not necessarily accompanied by complete loss of cell-cell contact. Rather, cohorts of cells migrate as cohesive sheets or as linked cells in single file (the so called 'Native American file'). This is certainly the case in malignant neoplasia of the breast (Pierson and Wilkinson, 1990). Why then should so many breast cancer cells and tumors have lost E-cadherin mediated adhesion if it may not be absolutely required for local invasion? Another important step in carcinoma progression is the movement of tumor cells or emboli to local lymph nodes or distant sites via

the lymphatic or venous circulation. In some situations it is possible that, in order to enter the fluid in a lymph vessel or vein, tumor cells must be detached from the primary tumor mass either as individuals or as aggregates by the laminar flow imposed by the circulatory system. In this case, the physical strength of homotypic cell adhesion may be a significant determinant in the ability of a tumor cell to enter the circulation.

In the present study we test the hypothesis that defects in E-cadherin-mediated adhesion result in a reduction in cell-cell adhesion strength which in turn leads to an increased likelihood of cells detaching from a tumor mass when exposed to lymphatic, venous or arterial flow. In order to exert tensile and shear forces on cell-cell contact sites, laminar flow was imposed on cell aggregates which were adherent to a planar substratum. The time course of the deformation and disaggregation response of the aggregates was recorded at a wide range of flow rates. The cells used in these assays included E-cadherin positive epithelial cells, cells with known defects in E-cadherin expression or function as well as cell lines transfected with E-cadherin. The assays showed that defects in the expression or function of E-cadherin or associated molecules significantly reduces the physical strength of homotypic cell-cell adhesion. We measure for the first time the strength of E-cadherin mediated adhesion and, importantly, show that the shear stresses required to disaggregate E-cadherin negative cells correspond closely to those found in a lymphatic vessel or capillary.

MATERIALS AND METHODS

Cells

The cell lines used in the experiments were E-cadherin positive normal human breast epithelial cells (MCF-10A) (Soule et al., 1990), E-cadherin positive weakly invasive human breast carcinoma cells (MCF-7), E-cadherin negative poorly invasive human breast cancer cell line SKBR3, E-cadherin negative highly invasive human breast carcinoma cells (HS578T, BT549; Sommers et al., 1991), E-cadherin and control transfected HS578T, BT549 cells (HS-Ecad, BT-Ecad; Sommers et al., 1994), E-cadherin transfected mouse L-cells (L-Ecad; Sommers et al., 1994), E-cadherin positive, α -catenin negative human colon cancer cell clone A (Breen et al., 1993). E-cadherin negative human colon cancer cell RKO, and E-cadherin and control transfected RKO cells (RKO-Ecad, Breen et al., 1995). Following 3 days in culture confluent cultures of cells were trypsinized with 0.025% trypsin in the presence of 5 mM Ca^{2+} . The resulting suspension of single cells and small aggregates was washed and resuspended in 5 ml of DMEM containing 5% FBS at a concentration of 2×10^6 cells/ml and maintained at 37°C in a humidified CO₂ incubator for 2-4 hours to regenerate cell surface proteins. All experiments were performed at 32°C within 4 hours of trypsinization.

Flow chamber

A parallel flow chamber of uniform width was used in the laminar flow assays (Chien and Sung, 1987). The chamber consists of (a) an upper plate having appropriate opening for the delivery of the fluid into and out of the channel, (b) a gasket with an opening in the form of a channel, (c) a transparent bottom plate (grade no. 1 coverslip) and (d) top and bottom stainless steel cover plates with observation slots. The bottom plate, the gasket, and the base plate are fastened between the cover plates. The entry port of the chamber is connected through a valve and teflon tubing to two syringes, one filled with cell suspension and the other filled with suspending medium. Before use

in the flow chamber glass coverslips were coated with laminin (10 $\mu\text{g}/\text{cm}^2$) or collagen type I (10 $\mu\text{g}/\text{cm}^2$) as described earlier (Tozeren et al., 1994).

A syringe pump (Harvard Apparatus) was used to pump medium into the chamber at specified flow rates. The shear stress on the bottom plate of the chamber along the direction of flow, τ (dyn/cm^2), was evaluated using the following equation, assuming Poiseuille flow:

$$\tau = 6\mu Q/h^2 w. \quad (1)$$

where μ (0.01 $\text{dyn}\cdot\text{s}/\text{cm}^2$) is the viscosity of the medium, Q (cm^3/s) is the flow rate, h is the gap thickness of the channel (0.012 cm) and w (1 cm) is the width of the chamber (Chien and Sung, 1987).

Laminar flow assays

Laminar flow assays were initiated by placing the flow chamber on the stage of an inverted microscope (Diaphot, Nikon Inc., Garden City, NJ) equipped with 10 \times and 40 \times Hoffman and brightfield objective lenses. The cell suspension was gently infused into the flow channel and cell aggregates allowed to interact with the matrix protein-coated glass coverslip for 20 minutes under static conditions. Flow was then initiated at $\tau = 1.75 \text{ dyn}/\text{cm}^2$ and the flow rate increased at 30 second or one minute intervals up to a maximum value of $\tau = 100 \text{ dyn}/\text{cm}^2$.

A video camera (DAGE-MTI) was attached to the side port of the microscope to record the deformation/disaggregation response of cell aggregates to imposed laminar flow. The times were displayed on the video monitor with a data mixer (Vista Electronics, La Mesa, CA) and the length and width of the cell aggregates before and during flow were determined using a position analyzer mixer (Vista Electronics, La Mesa, CA) that provided a digital readout proportional to the distance between two sets of vertical and horizontal lines.

Flow-induced disaggregation of both small (2-6) cells and large aggregates were recorded. Large aggregates were defined as those whose largest dimension before the imposition of flow was 70-140 μm . Large carcinoma cell aggregates typically contained multiple layers of cells with many cells adherent to neighboring cells but not to the planar substratum.

A detachment event was said to occur when a single cell or a small cell aggregate detached from the parent aggregate in response to the imposed flow. In each experiment with a large aggregate, the number of detachment events during a one minute interval of infusion at a constant level of shear stress was determined. The total number of disaggregation events performed with each cell type varied between 8 and 14. The mean and standard deviation of the number of detachment events were computed as a function of the fluid shear stress imposed on the laminin or collagen-coated glass coverslip. The mean value was denoted as the frequency of disaggregation.

RESULTS

Flow-induced disaggregation of large cell aggregates

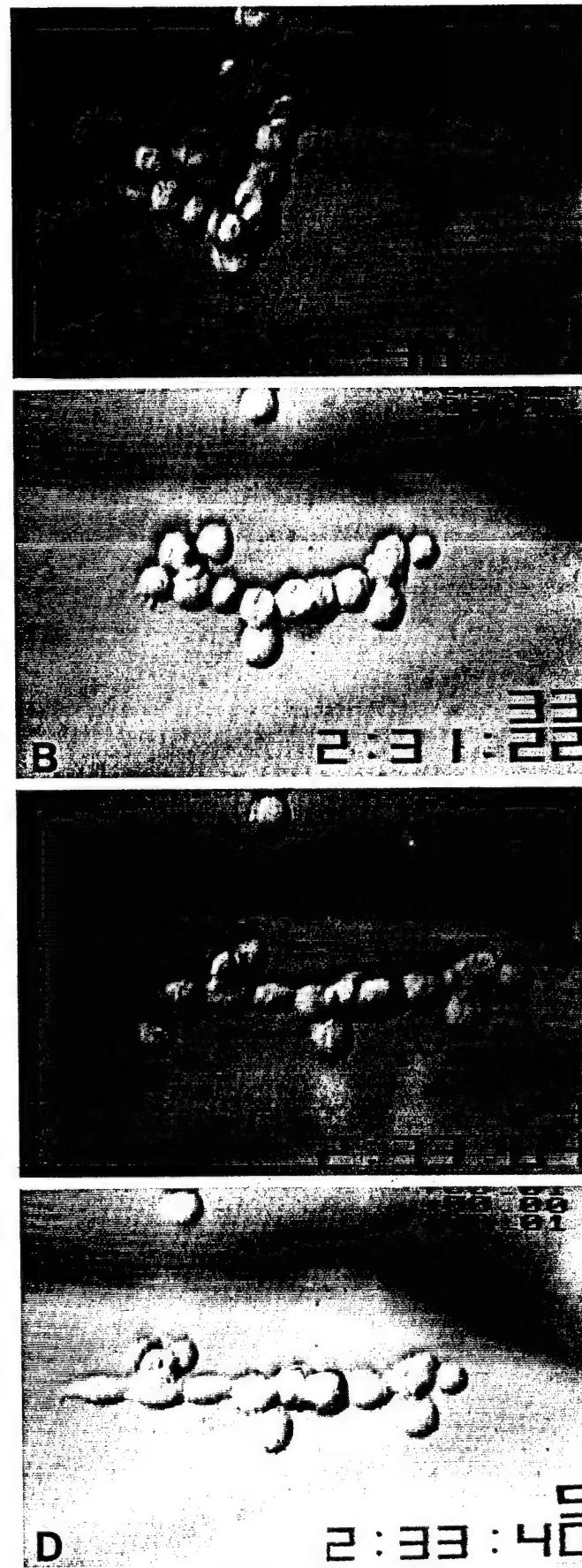
Laminar flow was imposed on large aggregates which were incubated on the bottom plate of the flow chamber for 20 minutes under static conditions. The coverslip was coated with either laminin or collagen depending on the cell type. Pilot experiments showed that MCF-10A and clone A cells attached more firmly to laminin and that MCF-7, HS578T and BT549 cells attached better to collagen type I. Following the attachment period, a few cells at the bottom of the aggregates formed adhesive contacts that were strong enough to resist detachment by flow. However, most of the cells in the aggregates were not in contact with the substratum and it is these cells that could

be detached (or could not be detached) from one another by flow. In this system, laminar flow will impose force on cell-cell contact sites only if some cells in the aggregate are anchored to the substratum. In this case it is clear that strength of cell-substratum adhesion of those cells which are in contact with the substratum must be strong enough to allow disaggregation without the aggregate detaching from the substrate as a whole. The videomicrographs (particularly Figs 1, 2 and 7) clearly show that even the small aggregates reoriented rapidly in response to the imposition of flow showing that cell-substratum attachment is not involved in the stretching response of the cells to shear forces. Generally, aggregates were anchored to the substrate through one or two cells, which remained attached to the substratum even after the rest of the aggregate completely disaggregated in response to flow. Cells that did become detached from one another were instantly swept away by the flow without any interaction with the substratum, indicating that cell-matrix adhesion did not contribute significantly to the observed phenomena.

MCF-7 and MCF10A cells which were pre-treated for several hours with low calcium medium (50 μ M) or with antibodies to E-cadherin prior to laminar flow assays did not form aggregates of measurable adhesive strength (not shown). In other studies using some of the same cells we demonstrate that for those cells with functional E-cadherin-mediated adhesion the ability to form aggregates is lost when cells are exposed to low calcium medium or antibodies to E-cadherin (Sommers et al., 1991, 1994). The E-cadherin positive cells used in these experiments do not form strong aggregates under these conditions, consequently cell-cell adhesion strength is very low and cell aggregates disintegrate as they are being infused into the flow chamber. Preformed aggregates exposed to low calcium medium in the flow chamber exhibited much weaker cell-substratum adhesion and many of the aggregates detached from the substratum as a whole at low shear forces. In two other cell types (L-cells and RKO cells) transfection of E-cadherin restored strong cell-cell adhesion whereas neo transfectants behaved as controls. These E-cadherin transfected cells also do not form aggregates in low calcium medium or in the presence of antibodies to E-cadherin (see also results in Sommers et al., 1994; Breen et al., 1995).

The fluid shear stress applied to the coverslip ranged from 2.5 dyn/cm² to 100 dyn/cm². At low flow rates, aggregates of E-cadherin positive MCF-10A or MCF-7 cells rapidly aligned in the direction of flow in order to reduce fluid drag (Fig. 1). At high flow rates, these aggregates deformed extensively, physically straining cell-cell contact sites (Figs 1, 2). However, cells or small aggregates could not be detached from the aggregates of MCF-10A or MCF-7 cells despite the imposition of high flow rates ($\tau = 100$ dyn/cm²; Table 1). In a few instances aggregates detached as a whole from the coverslip at high flow rates.

Fig. 1. Sequence of video-micrographs showing the typical deformation response of MCF-10A cells to imposed laminar flow. The numbers at the bottom of the screen represent hour, minute, second, and tens of milliseconds. Flow was initiated at 2:30:00 at $\tau = 1.75$ dyn/cm² and was incrementally increased every 30 seconds such that the shear stress τ took the values 1.75 (A), 3.5, 7.0 (B), 10.5, 14, 21, 35 (C) and 50 dyn/cm² (D). The figure shows that the string of MCF-10A cells orient in the direction of flow and deform extensively but do not detach from each other.



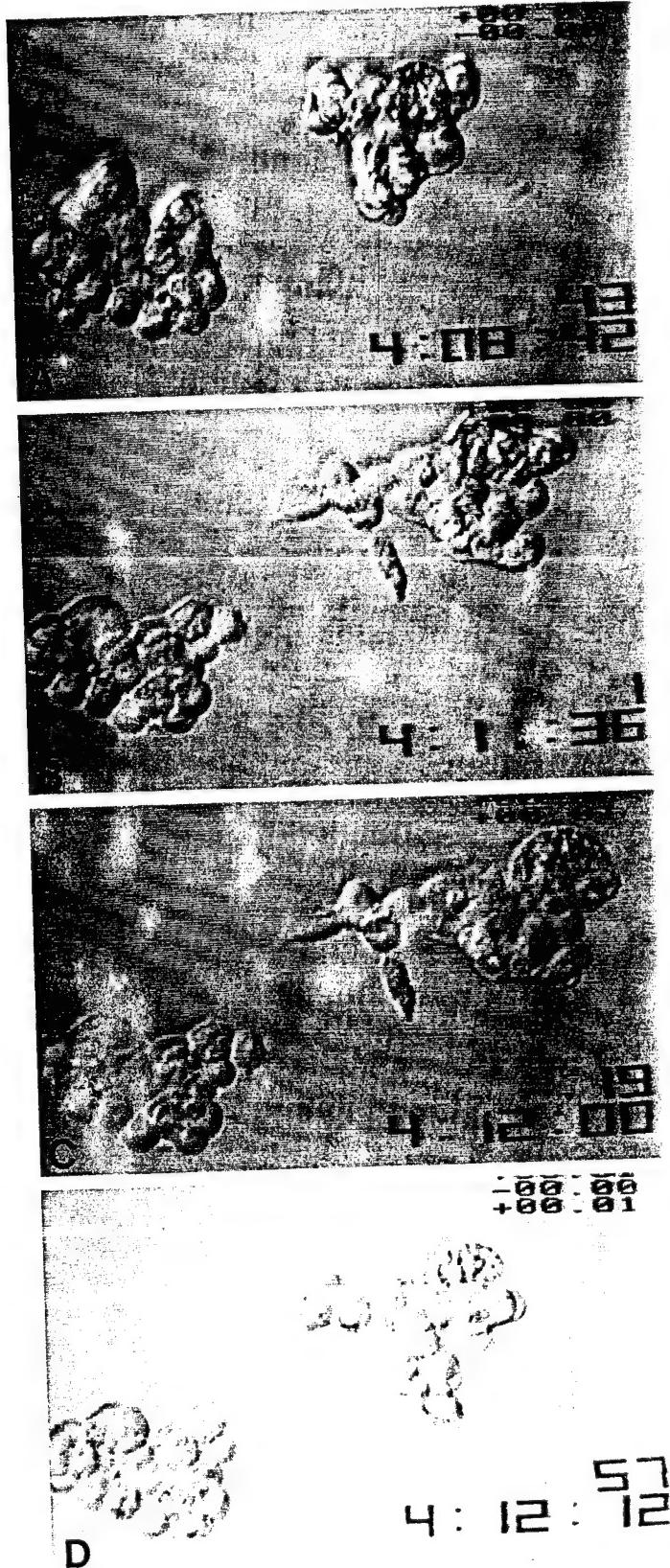


Fig. 2. Sequence of video-micrographs showing the typical deformation response of large aggregates of MCF-7 cells to imposed laminar flow. Flow was initiated at 4:08:00 at $\tau = 1.75 \text{ dyn/cm}^2$ and was increased every 30 seconds such that the shear stress τ took the values 1.75, 3.5, 7.0, 10.5, 14, 21, 35 dyn/cm^2 (B,C). The flow ceased at 4:12:02 and the aggregate returned to its original configuration in 10 seconds (D).

Table 1. Relationship between E-cadherin protein expression and E-cadherin function

Cell	E-cadherin	E-cad function	τ for disagg.	Invasiveness
MCF-10A	+	+	>100 dyn/cm^2	-
MCF-7	+	+	>100 dyn/cm^2	+
T47D	+	+	>100 dyn/cm^2	+
SKBR3	-	-	<7 dyn/cm^2	+
HS578T	-	-	<7 dyn/cm^2	+++
BT549	-	-	<7 dyn/cm^2	+++
RKO	-	-	<7 dyn/cm^2	+++
L949	-	-	<7 dyn/cm^2	++
HS578T-Ecad	+	-	<7 dyn/cm^2	+++
BT549-Ecad	+	-	<7 dyn/cm^2	+++
L949-Ecad	+	+	7-100 dyn/cm^2	+
RKO-Ecad	+	+	>100 dyn/cm^2	+
Clone A	+	-	>7 dyn/cm^2	++

The relationship was assessed by the presence or absence of Triton-insoluble E-cadherin at points of cell-cell contact and/or the ability of transfected E-cadherin to mediate a morphological change (Sommers et al., 1991, 1994; Breen et al., 1993, 1995), shear stress forces for disaggregation, and invasiveness. The invasive characteristics of these cells have been described previously (Sommers et al., 1991, 1994; Breen et al., 1993, 1995).

detached from the parent aggregate at low levels of fluid shear stress ($\tau = 2.5 \text{ dyn/cm}^2$).

The flow-induced detachment of cells and cell aggregates from the parent aggregate is a stochastic process that not only depends on the applied fluid shear stress, the geometry of the cell aggregate and its orientation with respect to flow, but also on the number density and the physical strength of the bonds which act to keep the cells together. For these reasons a large number of disaggregation experiments were performed on cell aggregates of comparable size (70-140 μm) for each cell type. The frequency of detachment events from parent aggregates as a function of applied fluid shear stress (τ) for HS-578T and BT-549 cells is presented in Fig. 4. Both these cell types began to disaggregate at fluid shear stress levels found in lymphatics and in the circulation ($2.5-15 \text{ dyn/cm}^2$). The frequency of detachment events decreased with increasing shear stress because the number of cells available for detachment was reduced during the course of the experiments. Thus, although the E-cadherin negative highly invasive breast carcinoma cells used in the present study form large aggregates in the presence of calcium, the shear forces required to disaggregate these cell aggregates are quite low.

In similar experiments we found that all cells which were E-cadherin negative exhibited a similar detachment response to flow (Table 1). However, the inability of cell aggregates to remain intact in laminar flow was not restricted to aggregates of E-cadherin negative carcinoma cells. Table 1 shows that the E-cadherin positive colon carcinoma cell line clone A also disaggregates in response to low shear stresses. This cell is known not to express the E-cadherin-associated molecule α -catenin,

In contrast, cells from large aggregates of E-cadherin-negative HS578T and BT549 breast carcinoma cells disaggregated in response to the imposed laminar flow at low to moderate flow rates ($2.5 \text{ dyn/cm}^2 \leq \tau \leq 15 \text{ dyn/cm}^2$). Fig. 2 shows that individual cells and small BT549 cell aggregates

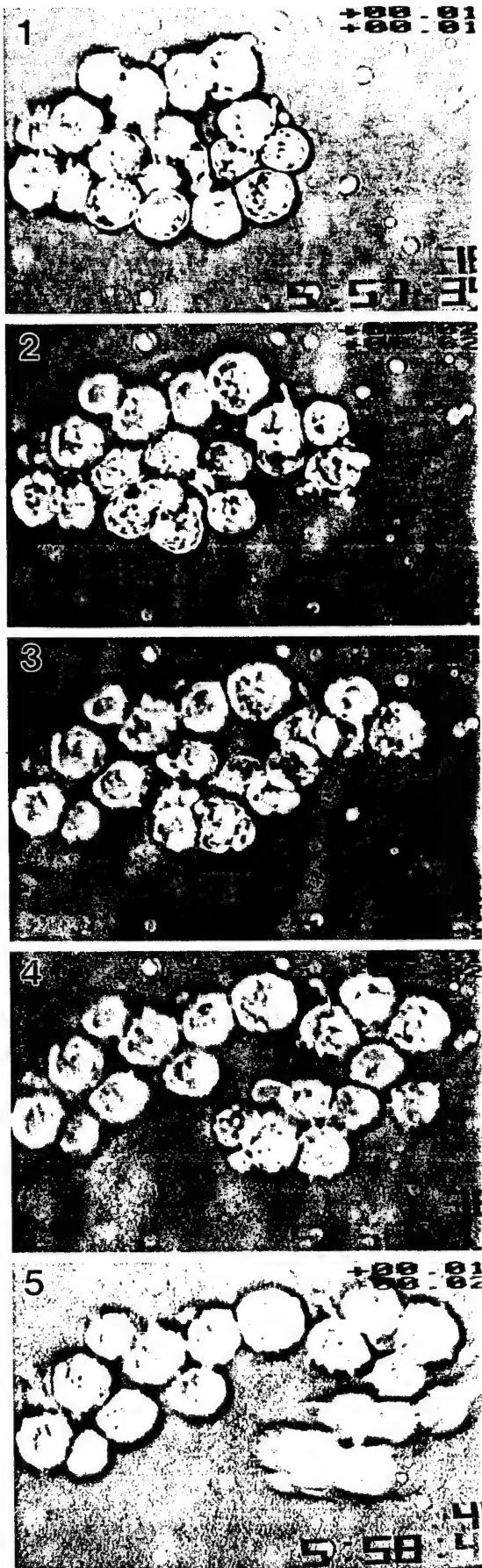


Fig. 3. Sequence of video-micrographs showing the flow-induced disaggregation of a large aggregate of E-cadherin-negative BT-549 cells. Flow was initiated at 5:57:00 at $\tau = 1.75 \text{ dyn/cm}^2$ and was increased every 60 seconds such that the shear stress τ took the values 2.5 dyn/cm^2 (1, 2), and 5.0 dyn/cm^2 (3, 4, 5).

defect that is likely responsible for the failure of aggregates of these cells to resist low shear stresses (Breen et al., 1993).

E-cadherin transfection prevents flow induced-disaggregation only when it is restricted to cell-cell contact sites

Transfection of BT-549 and HS-578T cells with E-cadherin cDNA did not change their flow-induced aggregation properties (Fig. 4) even though we had demonstrated previously that it could mediate specific aggregation with E-cadherin transfected fibroblasts (Sommers et al., 1994). Expression levels of E-cadherin protein in the transfected cells was similar to those of MCF-7 cells as judged by immunocytochemistry, western analysis and immunoprecipitation (Sommers et al., 1994). In contrast, aggregates of E-cadherin transfected RKO cells remained intact when exposed to high shear stresses (Fig. 5). Similarly, E-cadherin transfected L-cells acquired calcium-dependent cell-cell adhesion properties and their frequency of detachment was much lower at all shear stress levels than E-cadherin negative breast tumor cells (Fig. 5). As shown on several occasions by others, untransfected L-cells did not form aggregates in the presence or absence of calcium (Sommers et al., 1991, 1992, 1994). Similarly, E-cadherin transfected RKO cells do not form aggregates in low calcium medium or in the presence of E-cadherin antibodies (Breen et al., 1995). These results indicate that whereas E-cadherin expression is required for cell aggregates to resist high shear stress forces other factors also contribute to the ability of E-cadherin to mediate strong cell-cell adhesion. It is well known that E-cadherin is linked to the cell cytoskeleton through other molecules, β -catenin, α -catenin, γ -catenin and/or plakoglobin (see for review, Kemler, 1993). Alterations in the expression or phosphorylation state of these E-cadherin-associated molecules have previously been demonstrated to modulate E-cadherin mediated adhesion (Shimoyama et al., 1992; Hirano et al., 1992; Matsuyoshi et al., 1992). The two E-cadherin transfected invasive breast cancer cell lines used in the present study have elevated levels of tyrosine phosphorylated β -catenin and reduced plakoglobin levels (Sommers et al., 1994). In these cells the transfected E-cadherin is not restricted to cell-cell contact sites and is largely Triton soluble. In contrast, exogenous E-cadherin expressed in MCF-7 cells and in L-cells becomes restricted to cell-cell contact sites and is Triton insoluble in these areas (Sommers et al., 1994). Similarly, Triton insoluble E-cadherin is expressed at cell-cell contact sites in the E-cadherin transfected colon carcinoma cell line RKO (Fig. 6). Therefore the ability of cell aggregates to remain intact in laminar flow not only depends upon E-cadherin expression but also on the presence of a Triton-insoluble form of E-cadherin at cell-cell contact sites.

The role of E-cadherin expression in the physical strength of cell-cell contact sites

The capacity of cell-cell contact sites to resist external tensile forces was investigated by determining the deformation response of string-shaped aggregates to imposed laminar flow. Laminar flow imposed on MCF-10A chains led to extensive

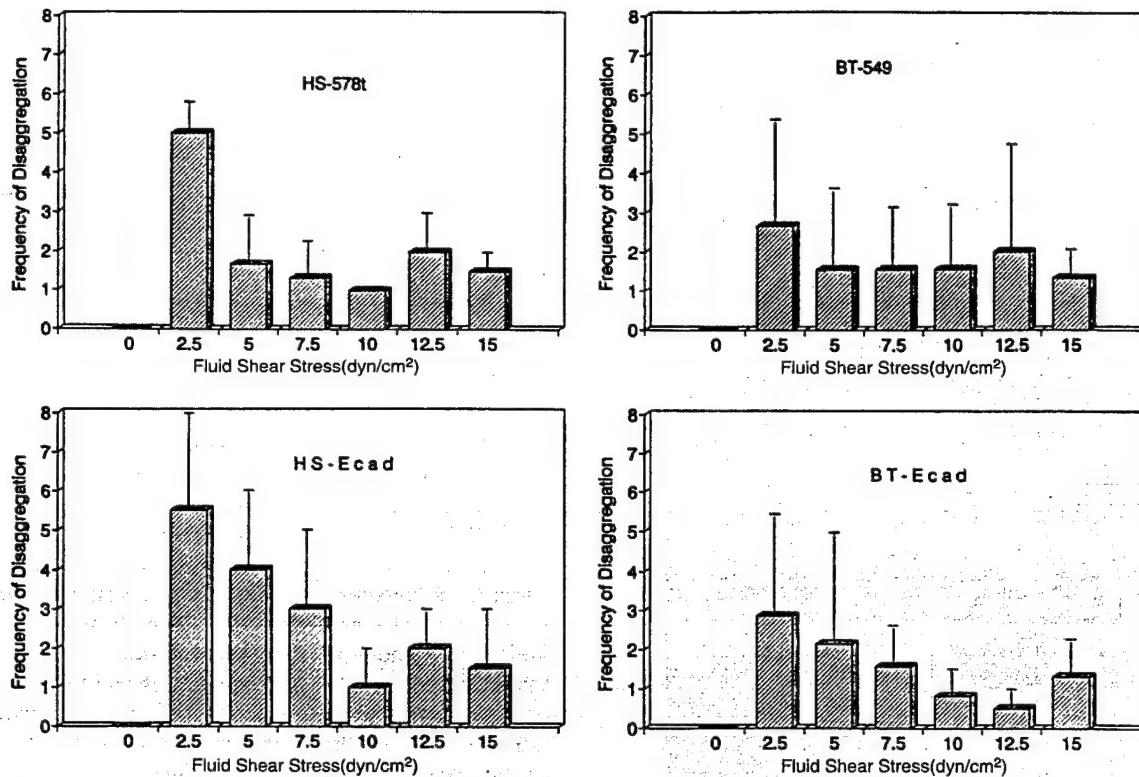


Fig. 4. Frequency of disaggregation of BT-549, BT-Ecad, HS-578T and HS-Ecad cell aggregates in response to applied fluid shear stress. The bars and vertical lines indicate the mean values and the standard deviation of the number of detachment events observed during 60 seconds of flow at a specified shear stress. The total number of experiments was 12. The disaggregation response of aggregates of control (BT-hyg and HS-hyg) cells was similar to that of untransfected cells (not shown).

cell elongation in the direction of flow (Figs 1, 7). As shown in the free body diagram (see Fig. 10) these cells were approximately under uniaxial tension loading. The cell aggregates remained attached to the substratum through a single cell at a few focal contacts and the adhesion contacts between cells could not be broken at levels of fluid shear stress greater than those found in arteries ($\tau = 100 \text{ dyn/cm}^2$). Flow-induced cell elongation became more pronounced with increasing shear stress and with relative position within the string of cells.

A measure for the extent of cell deformation in the direction of flow is the ratio of instantaneous cell length in the direction of flow (L) to the corresponding length before the imposition of flow (L_0). The deformation index (L/L_0) for three individual cells in different MCF-10A strings was plotted in Fig. 8 as a function of the tensile force exerted on each cell (F_{jm} , see the free body diagram in Appendix). This tensile force was estimated by using the known mathematical solutions of flow past strings of spheres or spheroids (Gluckman et al., 1971) as described in Appendix 1. Fig. 8 shows that MCF-10A cells elongated in the direction of flow as much as 60% under the

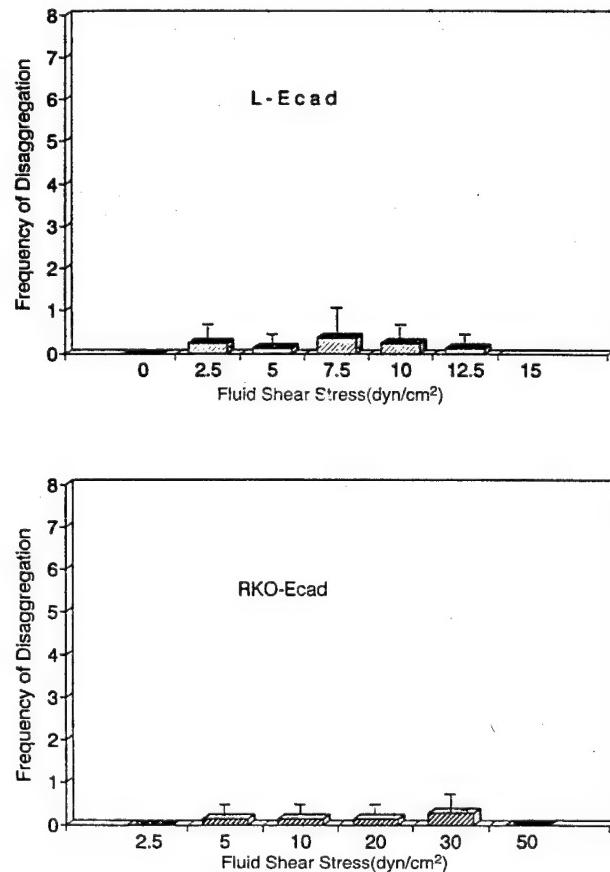


Fig. 5. Frequency of disaggregation of RKO-Ecad and L-Ecad cell aggregates in response to applied fluid shear stress. The bars and vertical lines indicate the mean values and the standard deviation of the number of detachment events observed during 60 seconds of flow at a specified shear stress. The total number of experiments in each case was seven. Aggregates of non-transfected and control RKO transfectants (RKO-neo) disaggregated as they were infused into the flow channel. Untransfected and control (L-neo) L-cells did not form aggregates under the conditions used in the experiments.

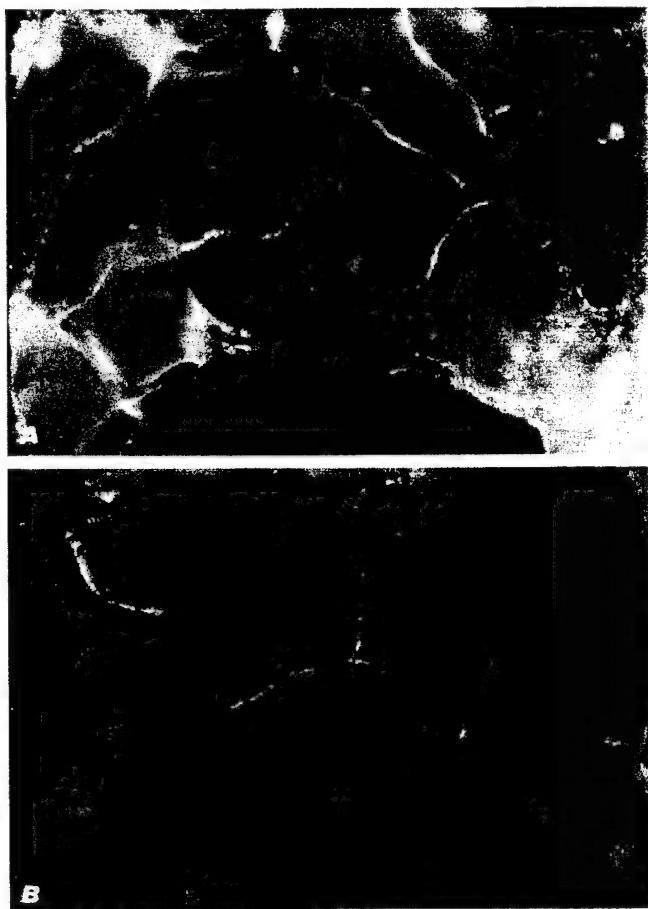


Fig. 6. Triton insoluble E-cadherin is present at cell-cell contact sites in E-cadherin-transfected RKO cells. RKO cells transfected with E-cadherin were immunostained for E-cadherin before (A) or after (B) extraction with Triton X-100 as described previously (Sommers et al., 1994).

action of tensile forces in the order of 10^{-3} dyn. The deformation response of these cells is elastic, as the string of cells returned to their undeformed configuration within a few seconds following the cessation of flow (Fig. 7). The force-deformation response of MCF-7 breast carcinoma cells was similar to that of MCF-10A cells (Table 2).

Fig. 9 shows the deformation and disaggregation response of a typical HS-578T doublet exposed to laminar flow. The cells started to detach from each other at $\tau = 7$ dyn/cm². The tensile force that caused detachment in this case (F_{22}) was estimated, using equations (2) to (5) in Appendix, as 10^{-5} dyn, a value that is two orders of magnitude smaller than the external force resisted by MCF-10A cell-cell contact sites. BT-549 cells also exhibited a weak adhesive contact (Table 2). Fig. 9 also shows that the HS-578T cell disaggregation was preceded by the formation of a tether between the two cells, a phenomenon that was also frequently observed during the flow-induced detachment of clone A and BT-549 cells.

DISCUSSION

In this study laminar flow assays were used to investigate the forces involved in homotypic cell-cell adhesion. Laminar flow

Table 2. Biophysical parameters of homotypic cell-cell adhesion

Cell type	Flow (dyn/cm ²)*	Tensile contact force (dyn) [†]	Longitudinal stretching [‡]
MCF-10A	>100	> 5×10^{-3}	>60%
MCF-7	>70	> 2×10^{-3}	>60%
HS-578T	>2.5	$\approx 10^{-5}$	Tethered
BT-549	>2.5	$\approx 10^{-5}$	Tethered

*Fluid shear stress that leads to disaggregation.

[†]Tensile force resisted by contact sites.

[‡]Longitudinal stretching before cell detachment.

was imposed on aggregates of cells that were adherent to a laminin or collagen-coated coverslip. The shear flow past aggregates exerted large forces on some of the cell-cell contact sites in the aggregate. The results indicated that cell-cell adhesion strength is severely compromised in E-cadherin negative carcinoma cells and that E-cadherin expression is a necessary but not sufficient condition for firm cell-cell adhesion. Cells which expressed E-cadherin in a Triton-insoluble form at cell-cell contact sites resisted disaggregation when exposed to shear stress forces in excess of 100 dyn/cm². In contrast, E-cadherin negative cells or cells in which E-cadherin was present as a diffusely distributed Triton-soluble form detached from one another at values of fluid shear stress comparable to those found in lymphatic and post-capillary blood venules. Consistent with these experimental observations the external forces resisted by adhesive contacts between E-cadherin positive MCF-10A cells were at least two orders of magnitude larger than those between E-cadherin negative breast carcinoma cells.

Transfection of E-cadherin into HS-578T and BT-549 cells does not alter their morphology or invasive properties (Sommers et al., 1994) and we show in this study that it has no effect on the disaggregation response of these cells to flow. However, the absence of a Triton-insoluble pool of E-cadherin in the transfected carcinoma cells points to a defect in E-cadherin interaction with the cytoskeleton (Sommers et al., 1994; Ozawa et al., 1990; Nelson et al., 1990). It is known that these particular invasive breast carcinoma cells have a defect in the expression or function of the cadherin associated molecules β -catenin and plakoglobin (Hirano et al., 1992; Matsuyoshi et al., 1992; Shimoyama et al., 1992; Sommers et al., 1994). The inability of these cells to link transfected E-cadherin to the cytoskeleton probably explains the failure of E-cadherin transfection to alter the disaggregation response of these cells in the present study. In order to rigorously test the contribution of E-cadherin-mediated adhesion to the resistance to disaggregation forces we transfected the mouse fibroblast cell line L-949 with E-cadherin. This line has previously been demonstrated to express several cadherin-associated molecules and to link the transfected cadherin to the cytoskeleton (Ozawa et al., 1990; McNeill et al., 1990). E-cadherin transfected L-cells acquired calcium-dependent cell-cell adhesion and had disaggregation properties in response to shear, similar to those of E-cadherin positive normal breast and non-invasive breast tumor cells (Fig. 4). Although a small number of E-cadherin transfected L-cells could be detached by shear forces the frequency of detachment was 20 fold less than E-cadherin negative tumor cells. As shown on several occasions by others,

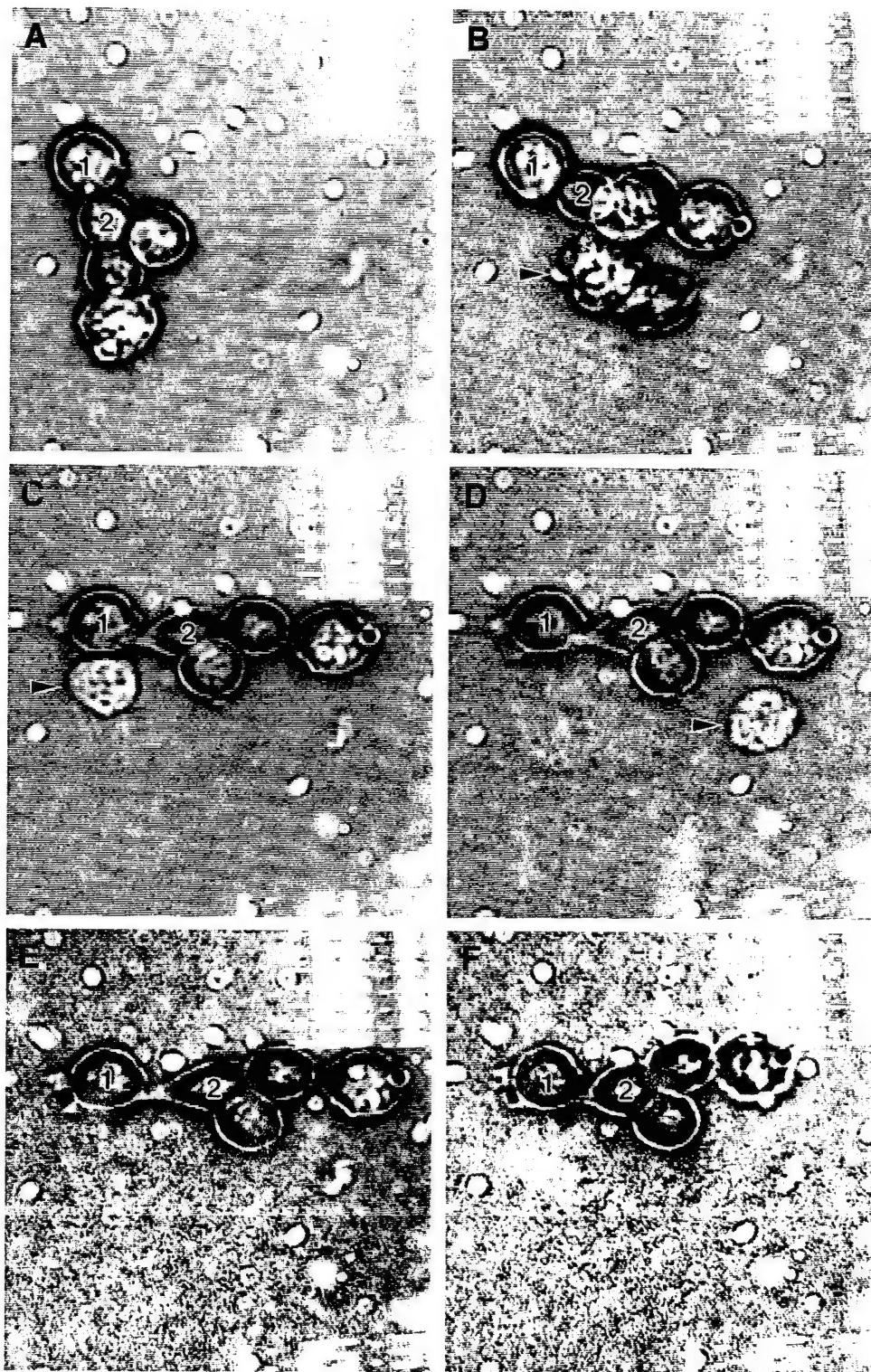
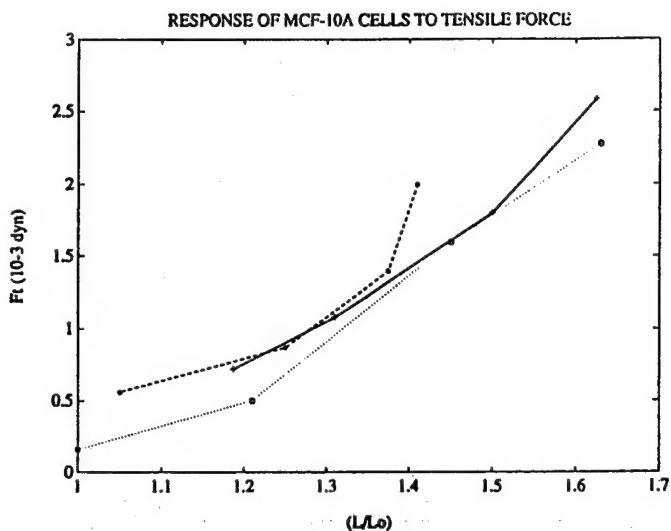


Fig. 7. The effect of shear stress on the orientation and deformation of a small aggregate of E-cadherin positive MCF-10A cells. The fluid shear stress on the laminin-coated coverslip corresponding to micrographs A-F was 0, 7, 35, 70, 100 and 0 dyn/cm^2 , respectively. Note the longitudinal stretching between cells 1 and 2. The arrowhead indicates a cell transiently interacting with the substratum.

untransfected L-cells did not aggregate significantly in the presence or absence of calcium (McNeill et al., 1990; Ozawa et al., 1990; not shown). Immunocytochemistry revealed a Triton-insoluble pool of E-cadherin at points of cell-cell contact in aggregates of non-invasive breast tumor cells and E-cadherin transfected L-cells indicating that a strong linkage had been established with the cytoskeleton (Sommers et al., 1994; Ozawa et al., 1990; Nelson et al., 1990). Another E-cadherin

negative carcinoma cell line that responds to E-cadherin transfection by a marked change in morphology and motility properties was also used to investigate the role of E-cadherin in adhesion strength (RKO-Ecad, Breen et al., 1995). These cells form few aggregates of low adhesive strength before E-cadherin transfection (Table 1). Following transfection of E-cadherin into these cells they acquired disaggregation properties similar to those of E-cadherin positive epithelial cells such



as MCF-10A (Table 1). These results indicate that E-cadherin-mediated adhesion is largely responsible for the disaggregation properties of cells which express this molecule on the cell surface and which are able to link it appropriately to the cell cytoskeleton. It is possible that further strengthening of

Fig. 8. The deformation response of three typical MCF-10A cells to tensile force. The data shown were obtained in laminar flow assays on MCF-10 cell aggregates in the form of strings of cells. The tensile fluid force (F_t) acting on a cell was computed as described in the appendix. The deformation index (L/L_0) denotes the ratio of the cell length at a specified shear stress and time to that before the imposition of flow. The parameter L was measured 28 seconds after the imposition of flow at a given fluid shear stress.

adhesion may require the assembly of other epithelial cell-specific junctions such as desmosomes.

The physical strength of adhesion between two cells is likely to be dependent upon a number of factors, including the number of adhesion bonds per contact area, their spatial distribution, and linkage to the cytoskeleton. In epithelial cells E-cadherin is generally restricted to the actin-associated adherens junction which forms a belt within which the E-cadherin is presumably present at a high local density and linked to the underlying actin cytoskeleton. The physical strength of MCF-10A cell-cell adhesion is comparable to that between T-lymphocytes and their specific target cells and between phorbol-12-myristate-13-acetate-stimulated T-lymphocytes and planar membranes containing intercellular adhesion molecule-1 (Tozeren et al., 1992a,b; Sung et al., 1986). In these experi-

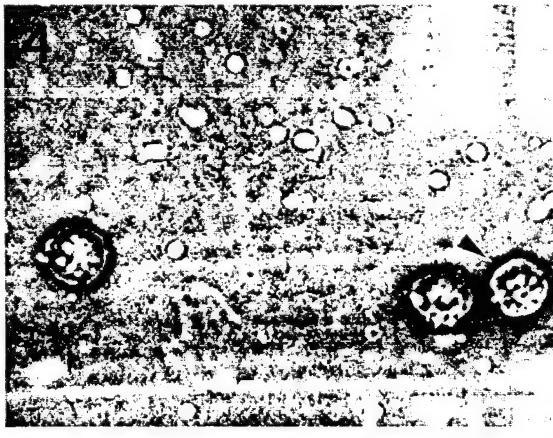
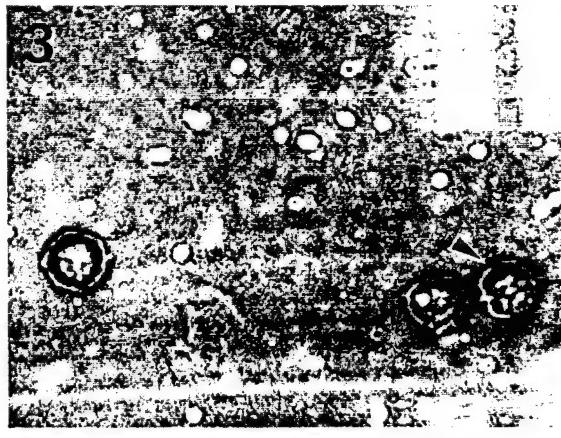
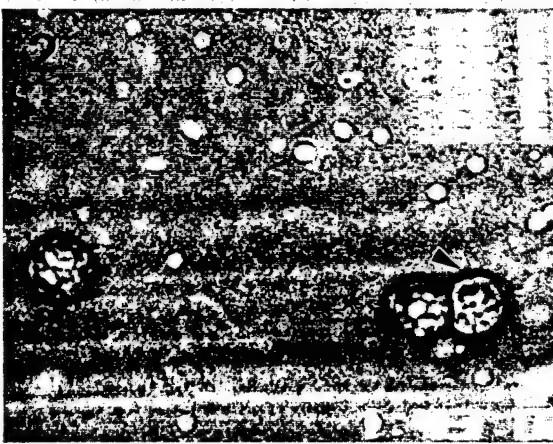
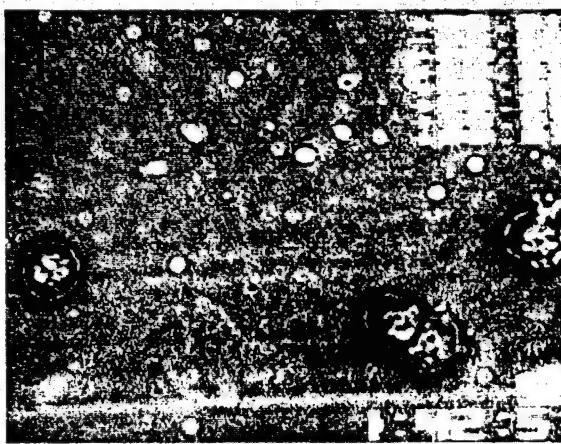


Fig. 9. The effect of fluid shear stress on the deformation and disaggregation of a HS-578T breast carcinoma cell doublet. The flow was imposed on the doublet adherent to a laminin-coated coverslip and was increased incrementally every 30 seconds. In micrographs 1-4 the fluid shear stress on the coverslip was 0, 7, 14 and 21 dyn/cm^2 . Note that the cell indicated by the arrowhead detaches from the adjacent cell without appreciable longitudinal stretching.

ments, the tensile forces acting on the adhesion sites were evaluated using a micromanipulation procedure in which cell couples were detached from each other using a micropipette attached to a pressure control system. The extent of MCF-10A elongation in response to tensile force is also comparable to that of T-lymphocytes under similar loading conditions suggesting similar bulk rheological properties (Tozeren et al., 1992a,b; Sung et al., 1986). MCF-10A cells retracted to their undeformed spherical configuration rapidly after the cessation of flow. This elastic behaviour may be due to metabolically regulated tension in the actin-rich submembrane cortical shell (Stossel, 1993). Alternatively, such elastic properties are inherent in the tensegrity model of cytoskeletal organization proposed by Ingber and co-workers (Wang et al., 1993).

The deformation response of E-cadherin-negative breast carcinoma cells to fluid forces is quite different from that observed with MCF-10A cells. At low to moderate flow rates tensile forces acting on contact sites between two cells typically results in the formation of tethers without appreciable change in cell shape. This suggests that although cell-cell adhesion does occur between these cells, the adhesive molecules which mediate it are not linked strongly to the cell cytoskeleton.

To our knowledge the present study is the first to use laminar flow assays to measure epithelial cell-cell adhesion strength. The use of such direct mechanical measurements coupled with the techniques of molecular and cellular biology may lead to a new understanding of the mechanochemical processes which govern many cellular events (Ingber, 1994). In this study such a combination of molecular and cellular manipulations together with biophysical approaches was used to investigate the role of E-cadherin in cell-cell adhesion. The results suggest that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. In these cells the relative strength of cell-substratum (endothelial cells

or extracellular matrix) and cell-cell adhesion is likely to determine whether a particular tumor would disaggregate in response to flow. This permits the speculation that once a breast or colon tumor infiltrates a blood or lymphatic vessel, E-cadherin negative cells may be detached by the flow and subsequently be captured by local lymph nodes or become lodged in distant capillary beds. These data, taken together with recent evidence that the E-cadherin-associated molecule β -catenin interacts directly with the tumor suppressor gene APC (Su et al., 1993), strongly suggest that alterations in the cadherin-based adhesion and signalling system not only affect the invasive capacity of tumor cells but may also influence contact-dependent growth control and metastasis.

APPENDIX

Evaluation of external forces acting on cell-cell boundaries

External forces acting on cell-cell contact sites in the direction perpendicular (tensile force) to the contact surface were computed by using data obtained from laminar flow assays on cell aggregates that were composed of a string of cells. Mathematical solution of axisymmetric flow past a finite chain of spheres each having radius R was considered (Fig. 10). Let m denote the number of spheres in the chain and integer j indicate the relative position of spheres along the chain such that $j = 1$ is the free end of the chain and $j = m$ is the end fixed to the substratum (Fig. 10). The drag force f_{jm} exerted by the surrounding fluid on the j sphere in the chain can be computed using the following equation:

$$f_{jm} = 6\pi\mu(U)(R)\lambda_{jm}, \quad (2)$$

where μ is the coefficient of viscosity of the surrounding fluid, U is the velocity of the uniform flow far from the chain of spheres, R is the radius of the identical spheres in the chain and the drag correction factor λ_{jm} is a function of the total number of spheres in a chain and the relative position of the sphere

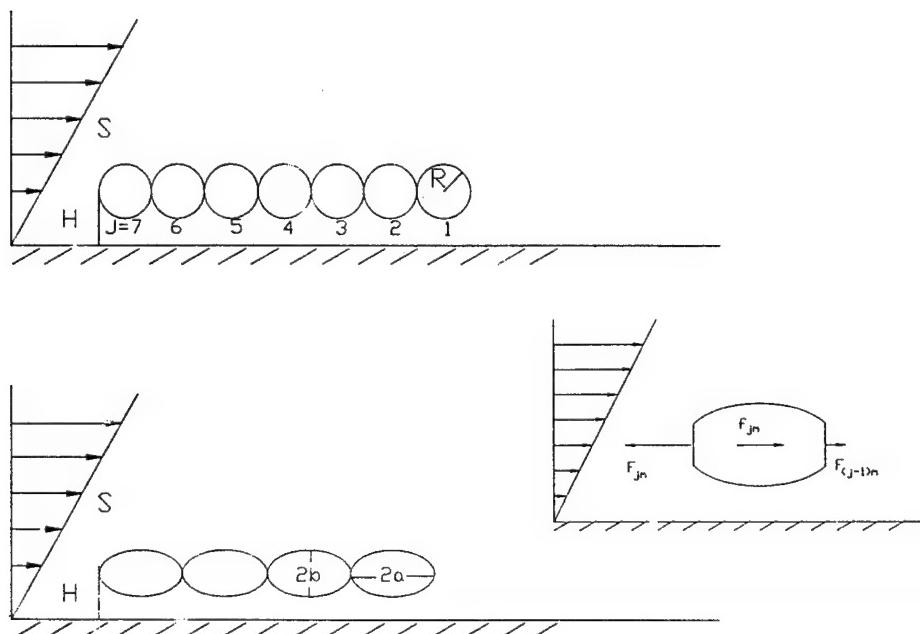


Fig. 10. Schematic diagram showing chains of spheres and spheroids fixed in simple shear flow near a planar boundary. Also shown is a free body diagram of a cell within the chain which indicates the forces exerted on the cell.

within the chain (Gluckman et al., 1971). $\lambda_{jm} = 1$ when $j = m = 1$, and $\lambda_{jm} = 0.65$ for $m = 2$ and $j = 1$ or 2. The solution for five or more chains indicates that the drag on the spheres located in the central portion of the chain changes little as the number of spheres in the chain is increased. As the end of the chain is approached, the drag on the spheres increases rapidly indicating a shielding effect. For example, in a seven sphere chain $\lambda_{17} = \lambda_{77} = 0.56$, $\lambda_{27} = \lambda_{67} = 0.28$, $\lambda_{37} = \lambda_{57} = 0.26$ and $\lambda_{47} = 0.25$ (Gluckman et al., 1971).

Let F_{jm} denote the total external force exerted by the surrounding fluid on the contact area between spheres j and $j + 1$ in a chain composed of m spheres (Fig. 10). The condition of the force balance indicates that:

$$F_{jm} = \sum_{j=1}^{m-1} f_{jm}. \quad (3)$$

This force is tensile in nature, that is, it will tend to stretch the bonds connecting the two cells in the direction perpendicular to the contact surface.

Gluckman et al. (1971) also provided Stokes flow solutions involving chains of prolate spheroids. A prolate spheroid is an axisymmetric body obtained by rotation of an ellipse along its long axis (Fig. 10). In this case of strings of prolate spheroids equation (2) is replaced by the following equation:

$$f_{jm} = 6\pi\mu(U)(b)\lambda_{jm}, \quad (4)$$

where b is the maximal radial distance along the minor axis of the prolate spheroid (Gluckman et al., 1971). In this case, the drag correction factor λ_{jm} depends not only on j and m but also on the ratio of the maximal length of the spheroid ($2a$) to its maximal diameter ($2b$). For two touching spheroids with $a/b = 2$, $\lambda_{12} = \lambda_{22} = 0.85$ the total drag force on a chain of spheroids is approximately equal to that of a chain of spheres having the same length and maximal radial distance (Gluckman et al., 1971).

In the experimental investigation presented here the cell chains were stationary not in uniform flow but in simple shear flow near a planar boundary. In order to use equations (2) and (4) in the actual experimental case, the velocity parameter U appearing in these equations was taken to be equal to the velocity of simple shear flow at the central axis of the string of cells:

$$U = (H)(S) = H\tau/\mu, \quad (5)$$

where H is the distance from the central axis of the cell chain to the planar membrane, S is the shear gradient of the simple shear flow and the wall shear stress τ is equal to the coefficient of friction m times the shear gradient S (Hsu and Ganatos, 1989). In this approximation, the effect of the planar wall on the drag coefficient factor λ_{jm} is not taken into account. The known solution concerning a sphere near a plane wall in simple shear flow indicates that wall effects may result in an increase in the drag force by as much as 60% (Goldman et al., 1967). Thus, the values provided here underestimate the external force resisted by MCF-10A cell-cell contact sites.

In actual computations, the wall shear stress τ was determined from the specified flow rate with the use of equation (1). The geometric parameters a and b were determined from the videotapes of the time course of deformation of the string of cells. The distance from the string center to the glass coverslip (H) was assumed for all cases to be equal to 1.5 times the maximal cell radius (b). The measured distance variations

required for the inverted microscope to clearly focus on the strings of cells with a 40 \times objective indicated that the assumed value of H cannot be different from the actual value by more than 50%. Because the tensile force is proportional to H , the order of magnitude of the estimate for the total drag force must be correct.

The string of cells attached at one end to the laminin-coated glass coverslip also had cells attached to the side of the chain (see Figs 1, 6). In such instances the contact forces between cells positioned upstream of the side-wise attached cell increased by the amount equal to the drag force acting on the side-wise attached cell. This force was computed by using the drag force coefficient factor for two equal spheres whose line of centers is perpendicular to the direction of imposed flow (Ganatos et al., 1978).

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Appendix 2

β -catenin associates with a kinase and is phosphorylated on serine in a breast cancer cell line

Robert J. Lechleider^{1,3}, Keith Orford², Connie Sommers², and Stephen Byers²

¹Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892-5055

²Department of Cell Biology, Georgetown University Medical Center, Washington, D.C.

20007

³To whom correspondence should be addressed

Running Title: β -catenin serine phosphorylation

Key Words: β -catenin, adhesion, serine phosphorylation, breast cancer, kinase

Abstract

β -catenin is an E-cadherin-associated molecule essential for the proper regulation of cell-cell adhesion. In mammalian cells, altered tyrosine phosphorylation of β -catenin is associated with increased invasiveness and decreased cell-cell adhesion strength. In the present study, immunoprecipitation and *in vitro* kinase analyses of detergent extracts from E-cadherin-negative invasive human breast cancer cells show for the first time that β -catenin associates with a serine kinase. Phosphoamino acid analyses demonstrate that serine phosphorylation of β -catenin by the associated kinase is many fold higher than tyrosine phosphorylation. Cell lines transfected with E-cadherin and with E-cadherin lacking the β -catenin binding region were used to demonstrate that β -catenin is associated with, and can be phosphorylated by an associated kinase in complexes both with and without E-cadherin. Immunoprecipitated β -catenin also exists as a serine phosphorylated protein in detergent extracts from metabolically labeled cells. These results indicate that serine kinase activity may regulate β -catenin functions in adhesion and signalling.

Introduction

In mammalian cells, β -catenin, a 97 kDa cytosolic protein, associates with cadherins, a family of transmembrane proteins responsible for mediating calcium-dependent homotypic cell-cell adhesion(1,2). β -catenin/cadherin complexes are anchored to the actin cytoskeleton at adherens junctions via α -catenin, a vinculin homolog, and other unknown molecules(1,3,4). The resulting detergent-insoluble complex is thought to be responsible for mediating strong cell-cell adhesion. Plakoglobin, a molecule closely related to β -catenin, also interacts with E-cadherin and is localized to cell-cell junctions(5-7). In addition to its role in modulating cadherin-based cell-cell adhesion, β -catenin can also associate with the product of the familial adenomatous polyposis coli (APC)¹ gene(8,9) suggesting a role in the regulation of cell proliferation. However, β -catenin complexes which contain both cadherin and APC do not exist indicating that β -catenin can associate with APC or cadherin but not both(8,9).

Phosphorylation of β -catenin may play a role in regulating its function. The Drosophila β -catenin homolog, armadillo (arm), requires the function of the serine-threonine kinase zeste-white 3 (zw3), a homolog to mammalian GSK3, for proper propagation of the signal generated by wingless (wg), the wnt-1 proto-oncogene homolog(10-12). In mammalian cells which overexpress certain tyrosine kinases, β -catenin can be phosphorylated on tyrosine(13-15). β -catenin tyrosine phosphorylation is increased in certain invasive but not non-invasive breast cancer cell lines(16) and increased β -catenin tyrosine phosphorylation is

¹Abbreviations used: Adenomatous polyposis coli, APC; phosphoamino acid analysis, PAA; *armadillo*, *arm*; *wingless*, *wg*; *zeste-white 3*, *zw3*; glycogen synthase kinase 3, GSK3; sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE

generally associated with decreased cell-cell adhesion and increased invasiveness. Tyrosine phosphorylated β -catenin continues to associate with E-cadherin and α -catenin but the complex as a whole may dissociate from the actin cytoskeleton(13,15). However, the physiologic role of tyrosine phosphorylation in regulating β -catenin activity is not known. Similarly, no role for serine phosphorylation in β -catenin function has been demonstrated. Although genetic data in Drosophila suggest that serine phosphorylation may play a key role in stabilizing the catenin-cadherin complex at the adherens junction, or play a pivotal role in the wnt-1 signaling pathway, it is not known if β -catenin is actually phosphorylated on serine or whether β -catenin complexes exhibit serine or tyrosine kinase activity.

In this report, we demonstrate for the first time the constitutive serine phosphorylation of β -catenin in a human breast cancer cell line. We also demonstrate that β -catenin immunoprecipitates exhibit serine-threonine kinase activity, and that this kinase is capable of phosphorylating β -catenin and associated proteins *in vitro*.

Materials and Methods

Cell culture: The generation of the BT-ECAD, containing a stably transfected mouse E-cadherin cDNA, and BT Δ , containing a truncated E-cadherin gene lacking the β -catenin binding domain, has been described previously(16). Cells were maintained in Dulbecco's modified Eagle's medium with 4000 gm/L glucose(Life Technologies), 100 U/ml penicillin and 100 μ g/ml streptomycin.

Immunoprecipitation and *in vitro* kinase assay: Confluent cultures in 150 mm dishes were washed twice in isotonic buffer and lysed for 15 minutes on ice in N-40 lysis

buffer(17) with 1 mM NaVO₄, 50 mM NaF, 20 µg/ml PMSF, 2µg/ml leupeptin and 1 µg/ml pepstatin and 1 µg/ml aprotinin. After clarification for 15 minutes at top speed in a microfuge at 4° C, lysates were pre-cleared with 100 µl protein A Sepharose (Pharmacia) and 20 µl normal rabbit serum twice for one hour. Protein content was measured by a colorimetric method (Bio-Rad). Equal amounts of protein (approximately 1 mg) were incubated with 2 µl of anti-β-catenin rabbit antiserum(18) or non-immune serum (BT 549 cells) or 15 micrograms of anti-E-cadherin (DECMA) rat ascites(19) or normal rat serum and 40 micrograms rabbit anti-rat IgG (BT-ECAD and BT delta cells) for 2 hours at 4° C. Immune complexes were precipitated with 10 µl protein A sepharose, washed 6 times in lysis buffer and once in kinase buffer(20 mM Pipes, pH 7.0, 150 mM NaCl, 1 mM NaVO₄, 50 mM Na). Immunoprecipitates were incubated in 10 µl of kinase mix (20 mM Pipes, pH 7.0, 2 mM MgCl₂, 10 mM MnCl₂, 50 mM Na, 1 mM NaVO₄, 10 uCi ³²P-γ-ATP (6000 Ci/mmol, NEN-Deepened)) for 20 minutes at room temperature, and the reaction stopped by the addition of 10 uL of loading buffer. Labelled proteins were analyzed by SDS-PAGE and autoradiography.

³²P-metabolic labeling and immunoprecipitation: Following starvation for 90 minutes in phosphate-free medium with 10% dialyzed fetal bovine serum, confluent 150 mm dishes of cells were labelled with 500 uCi/ml of ³²P- orthophosphoric acid (NEN- Deepened) for 3 hours. Immunoprecipitations were carried out as described above except that lysates were pre-cleared once with 100 µl of Protein A sepharose and 50 µl of normal serum and protein concentration was not determined. Following washes, immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

Phosphoamino acid analysis: Phosphoamino acid analysis was performed as described(17). Briefly, bands were excised from the gel, proteins were eluted with ammonium bicarbonate, trichloroacetic acid precipitated and acid hydrolyzed. PAAs were resolved by two-dimensional thin layer electrophoresis using a Multiphor II apparatus (Pharmacia), and exposed to x-ray film.

Results

Following metabolic labeling with ^{32}P -orthophosphate, three major bands are specifically immunoprecipitated by anti- β -catenin antibodies in BT 549 cells, which lack E-cadherin (Fig 1A, lane 2). The 97 kDa band in this system is β -catenin, as evidenced by reprecipitation after boiling the samples in SDS and re-incubating with antibody (data not shown) and consistent with previous data(16). The middle of the three prominent bands (102 kDa) is α -catenin. The identity of the upper band (120 kDa) is unknown but may be a form of cadherin other than E-cadherin since these cells do exhibit calcium-dependent adhesion even though they do not express E-cadherin (16). Metabolically labeled BT549 cells which have been stably transfected with the mouse E-cadherin gene also produce three major bands following immunoprecipitation with a monoclonal antibody to E-cadherin. In this case the 120 kDa species is E-cadherin, the 102 kDa species α -catenin, and the 97 kDa species β -catenin(16).

In addition to these three major bands, several other phosphorylated proteins are evident in β -catenin and E-cadherin immunoprecipitates. A high molecular weight (>200 kDa) phosphorylated protein is specifically immunoprecipitated by β -catenin antibodies but

not E-cadherin antibodies (Fig 1, Lane β -cat). This probably represents APC, shown previously to be associated with β -catenin in colon cancer cells(8,9). In the present study it is interesting to note that this protein is not found in E-cadherin immunoprecipitates even though β -catenin is present (Fig 1). A faint band is consistently present at approximately 130 kDa as well as several species in the 45-65 kDa range which are evident upon longer exposure (data not shown).

To determine if there is any difference in the phosphorylation state of β -catenin immunoprecipitated with anti-E-cadherin versus the total available β -catenin, we performed PAAs on the β -catenin bands from each immunoprecipitation. Phosphorylation of β -catenin from both the E-cadherin and β -catenin immunoprecipitates was exclusively on serine (Figure 1B and 1C). No tyrosine phosphorylation was observed even after prolonged exposure(data not shown). In previous studies using these cells tyrosine phosphorylation of β -catenin was only observed if cells were preincubated prior to lysis with hydrogen peroxide and vanadate (16). This implies the existence of an active tyrosine phosphatase which, when inactivated by pretreatment, allows visualization of tyrosine phosphorylation of β -catenin.

In order to determine if β -catenin directly associates with a kinase, we performed immunoprecipitation and *in vitro* kinase reactions. As shown in Figure 2A, β -catenin immunoprecipitates treated with 32 P- γ -ATP contained several specific proteins (compare NI (non-immune), with β -cat (anti- β -catenin)). β -catenin migrates as a 97 kDa phosphoprotein in both anti- β -catenin and anti-E-cadherin immunoprecipitates (Figure 2A, lanes 2 and 4), consistent with the results from metabolic labeling. The 102 kDa species is α -catenin. In the *in vitro* kinase experiments phosphorylated cadherin may be obscured by non-specific bands

present in all immunoprecipitates. High molecular weight proteins (>200 kDa) are phosphorylated in the anti- β -catenin but not anti-E-cadherin immunoprecipitates as was observed in the metabolically labeled samples. Similarly, a set of proteins is specifically phosphorylated in the 45-65 kDa range. The identities of these proteins are unknown. Anti-E-cadherin immunoprecipitates from BT Δ cells, which express a truncated version of E-cadherin that fails to bind β -catenin, do not contain any specifically phosphorylated bands (Fig 2A, last two lanes). The anti-E-cadherin antibody used (DECMA), immunoprecipitates truncated and full length E-cadherin equally well (data not shown). These results suggests that β -catenin specifically associates with a kinase, and that the kinase can phosphorylate other members of the β -catenin complex. Whether the kinase associates with β -catenin directly or through another protein cannot be determined from this experiment. However, the demonstration that no phosphorylated proteins could be demonstrated in E-cadherin immunoprecipitates from cells transfected with truncated E-cadherin suggests that regions of the E-cadherin cytoplasmic tail other than those involved in β -catenin binding do not associate with a serine kinase in the absence of β -catenin. It is not possible to ascertain from these data if the kinase/ β -catenin complex exists (or is active) only in the presence of a cadherin, or also occurs in the absence of β -catenin-cadherin interaction.

The phosphorylation state of the β -catenin phosphorylated *in vitro* was determined by PAA. As shown in Figure 2B, *in vitro* phosphorylated β -catenin is phosphorylated predominantly on serine with some threonine phosphorylation. These results indicate that β -catenin associates directly, or indirectly through another member of the complex, with a serine/threonine kinase that is able to phosphorylate β -catenin and other members of the

adherens junction complex *in vitro*. The total cellular β -catenin pool does not associate with an additional tyrosine kinase, as evidenced by the identical phosphoamino acid patterns of the total-cellular and cadherin-associated β -catenin.

Discussion

Although we and others have reported the tyrosine phosphorylation of β -catenin following inhibition of tyrosine phosphatases, we believe this to be the first report of phosphorylation on serine. Serine phosphorylation of β -catenin may play an important role in regulation of β -catenin localization or function. In Drosophila, the β -catenin homolog, the *arm* gene product, is an essential component of the *wg* signaling pathway(10). *arm* mutants display segment polarity defects similar to those displayed by *w* mutants (10). *wg* signaling is further regulated by the action of *zw3*, the Drosophila homolog of the serine-threonine kinase GSK3. Flies which lack *zw3* display a phenotype opposite to that of *arm* mutants(10). Peifer et al.(20) have proposed that *zw3*, the Drosophila GSK3 homolog, plays a role in maintaining *arm* stability during embryogenesis. The mammalian *wg* homolog, the proto-oncogene *wnt-1*, has been shown to increase cell-cell adhesion by stabilizing β -catenin or plakoglobin association with E-cadherin, suggesting that *wnt-1* may function through a similar pathway(11,12). Although no direct association of *zw3* with *arm* has been reported , rat *GSK3* can substitute for drosophila *shaggy*, a highly similar kinase active in the *Notch* signaling pathway(21). Taken together with our results, these data point to a possible *in vivo* association of β -catenin with a serine kinase of the *GSK* family.

Serine phosphorylation of β -catenin may play several roles in regulating mammalian adherens junction function. One study indicates a role for serine kinase activity in regulating the compaction of mouse embryos, an event dependent upon E-cadherin mediated adhesion(22). β -catenin serine phosphorylation may also be regulated by extracellular signals. For example, treatment of MCF7/6 cells with insulin results in increased E-cadherin-mediated cell-cell adhesion (23). It is tempting to speculate that this is related to the well-known, rapid effects of insulin on GSK activity which could result in changes in β -catenin serine phosphorylation and in turn influence cell-cell adhesion. Recent evidence also indicates that β -catenin protein expression and localization is regulated by retinoic acid².

These data together with information from other studies lead to the following working model. Firstly, cellular β -catenin levels are regulated by retinoids and perhaps other steroid receptor ligands. Secondly, β -catenin exists in three pools, a cytoplasmic pool, and two cadherin associated membrane pools, one immobilized to the actin cytoskeleton (detergent insoluble) and one not (detergent soluble)(1). Thirdly, β -catenin interacts with different proteins in the three pools and β -catenin stability and movement among the three pools is regulated by the balanced action of serine and tyrosine kinases(20). In this model, alterations in the expression or function of certain retinoid receptors and/or any of several serine and tyrosine kinases would have marked effects on β -catenin homeostasis. This could, in turn, influence not only cell-cell adhesion, but also, via the known interaction of cytoplasmic (but not cadherin-associated) β -catenin with the tumor suppressor gene APC, regulate cell proliferation.

²S.Byers, M.Pishvaien, M.Sporn, A. Tozeren, M.Anzano, and R.J. Lechleider, submitted

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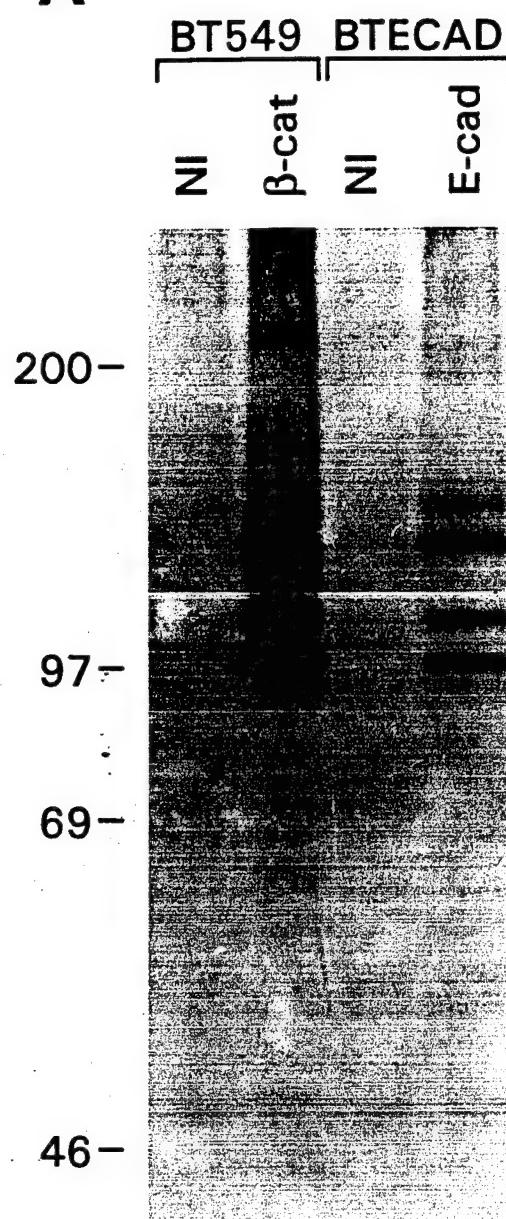
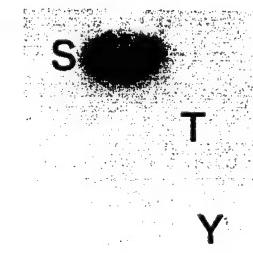
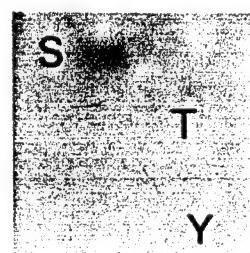
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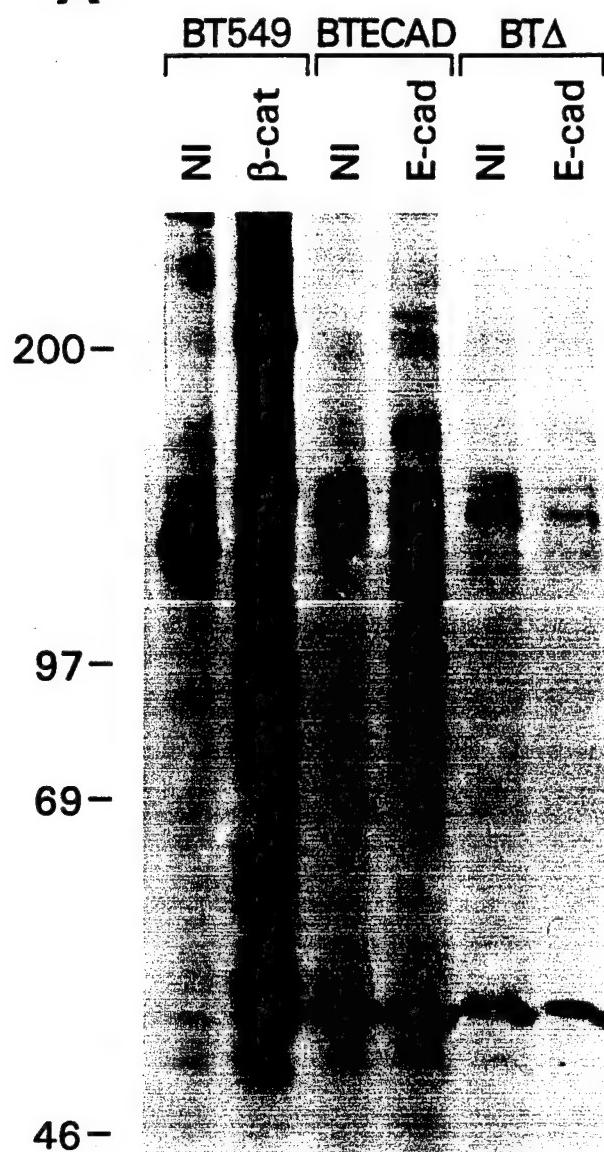
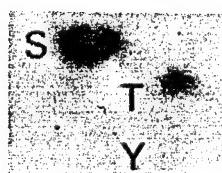
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Figure legends

Figure 1 β -catenin is phosphorylated on serine in metabolically labeled cells. (A) BT549 cells or BT-ECAD cells were metabolically labeled with ^{32}P -orthophosphate and immunoprecipitated with normal rabbit serum (NI), anti- β -catenin antibody (β -cat), normal rat serum (NI), or anti-E-cadherin (E-cad) antibody as indicated. Molecular weights are in kDa. Washed immunoprecipitates were analyzed by SDS-PAGE and autoradiography. PAAs were performed on the β -catenin bands from the (B) anti- β -catenin and (C) anti-E-cadherin immunoprecipitates. Expected positions of phosphoamino acids are indicated.

Figure 2. β -catenin associates with a serine/threonine kinase that can phosphorylate members of the adherens junction complex *in vitro*. (A) Lysates from the indicated cell lines were immunoprecipitated with antibodies as in Figure 1. Immunoprecipitates were washed, incubated with ^{32}P - γ -ATP, and analyzed by SDS-PAGE and autoradiography. Molecular weights in kDa at the left. (B) PAA of the β -catenin band from BT549 cells. Expected positions of phosphoamino acids are indicated.

A**B****C**

A**B**

1 **Retinoids Increase Cell-Cell Adhesion Strength, β -Catenin Protein**

2 **Stability and Localization to the Cell Membrane in a Breast Cancer Cell**

3 **Line. A Role for Serine Kinase Activity**

4

5 **Stephen Byers^{1,3,4}, Michael Pishvaian¹, Caroline Crockett¹, Christopher Peer³, Aydin Tozeren²,**
6 **Michael Sporn³, Mario Anzano³ and Robert Lechleider³**

7

8 **¹Department of Cell Biology and the Lombardi Cancer Center, Georgetown University Medical**
9 **Center, 3900 Reservoir Road, N.W. Washington D.C. 20007.**

10 **²Department of Mechanical Engineering, The Catholic University of America, Washington D.C.**
11 **20064**

12 **³Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892-5055**

13

14 **⁴To whom correspondence should be addressed. (202) 687-1813 (voice): (202) 687-1823 (fax)**

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16 **Keywords: Retinoid, cadherin, catenin, adhesion, kinase.**

17

18 **Short title: Retinoid regulation of β -catenin**

Abstract

In this study we show that a breast cancer cell line (SKBR3) which expresses no E-cadherin very low levels of β -catenin protein and exhibits a poorly adhesive phenotype in Matrigel, responds to retinoic acid (RA) by a marked increase in epithelial differentiation. Specifically, treatment of cells with all trans RA, 9-cis RA or a RA receptor α -specific ligand resulted in a large increase in cell-cell adhesive strength and stimulated the formation of fused cell aggregates in Matrigel. A retinoid X receptor-specific ligand was ineffective. Exposure of cells to RA for as little as 4 h was sufficient to maintain the adhesive phenotype for at least 4 days. The effects of RA required protein and RNA synthesis but were not mediated by factors secreted by stimulated cells, nor by direct cell contact and did not require serum. These RA-induced morphological effects were completely reversed by growing cells in 50 μ M Ca^{++} suggesting a mechanism involving a RA-induced increase in Ca^{++} -dependent adhesion. Consistent with this, β -catenin protein levels were markedly elevated in the RA-treated cells and β -catenin became localized to a Triton-insoluble pool at regions of cell-cell contact. No change could be detected in β -catenin steady state mRNA levels but RA did increase β -catenin protein stability. Treatment of cells with low calcium medium did not prevent the RA-induced increase in total β -catenin protein but did prevent its movement to a Triton-insoluble pool at the cell membrane. Among several kinase inhibitors, only the broad spectrum kinase inhibitor staurosporine and the protein kinase C inhibitor bisindolylmaleimide reversed the morphological changes induced by RA. Like treatment with low calcium medium, these inhibitors did not prevent the RA-induced increase in total β -catenin protein levels but completely prevented the movement of β -catenin to the cell membrane. These results, point to a role for β -catenin and serine kinase activity in mediating the action of RA in epithelial differentiation.

Introduction

Cadherin-mediated adhesion is fundamentally involved in the organization of epithelial tissues

3 during development, and manipulations of cadherin function result in profound disturbances of tissue

organization (Takeichi, 1990; Takeichi, 1991). In order for cadherins to function in cell-cell adhesion and

5 promote the formation of junctions several other associated molecules must be expressed (see (Kemler,

6 1993) for review). These molecules α , β and γ (plakoglobin) catenins, link the cadherins to the

underlying actin cytoskeleton and are probably involved in propagating adhesion-related signalling. The

8 importance of catenins in mediating adhesion and differentiation is underscored by the finding that the

9 expression of certain catenins is lost in malignant breast carcinoma cells and that the phosphorylation state

of β -catenin can also influence the transformed phenotype (Sommers et al. 1994b; Matsuyoshi et al.

11 1992).

In previous studies we showed that the expression of certain epithelial markers including E-

13 cadherin and the catenins was associated with the differentiation state of a series of breast cancer cell lines

14 (Sommers et al. 1991; Sommers et al. 1992; Sommers et al. 1994b). Generally, molecular markers of

epithelial differentiation were present in normal breast epithelial cells and in well differentiated breast

16 cancer cells but were reduced or absent in poorly differentiated highly invasive cells. Highly invasive

17 cells were also inevitably characterized by expression of the mesenchymal intermediate filament protein

vimentin (Sommers et al. 1994a). We also described a group of cell lines which were deficient in cell-cell

adhesion but which did not express vimentin and were not invasive. These cells were hypothesized to

represent an intermediate stage in a spectrum of malignant progression (Sommers et al. 1994a). When

21 antibodies to E-cadherin were added to well differentiated lines they underwent a morphological change

to an intermediate phenotype but did not become invasive. In certain instances exogenous expression of

"missing" E-cadherin or catenins into poorly differentiated invasive cancer cells renders the cells less invasive but in others cases it does not (Frixen et al. 1991; Hirano et al. 1992; Navarro et al. 1993). However, for the most part it is unlikely that the cell de-differentiation that accompanies malignant progression is a result of a single change in, cadherin expression or function. Indeed, this reasoning runs counter to the prevailing dogma that carcinogenesis is a multistage, polygenic disease (Fearon and Vogelstein, 1990; Cho and Vogelstein, 1992). This view of carcinoma as a disease which simultaneously affects several interactive genetic and signalling pathways naturally leads to the conclusion that agents used in the treatment of this group of diseases should also broadly affect multiple (perhaps even alternative or compensatory) pathways. Retinoids are clearly in this category.

Vitamin A and its derivatives (retinoids) are required to support many processes essential to the survival of eukaryotes. These include, vision, reproduction, hematopoiesis, bone development, various aspects of metabolism and pattern formation in development (see (Gudas et al. 1994) for review). Retinoids also regulate cell proliferation, epithelial differentiation and are effective in the treatment of several disease states including certain cancers (Gudas et al. 1994). With the exception of the retinaldehyde/rhodopsin mechanism involved in vision, the retinoids exert their action at the level of nuclear receptors which, when activated by retinoids, can modulate the expression of target genes by binding to specific responsive elements (HREs) in the promoter (Mangelsdorf et al. 1993). Once activated, this machinery, directly or indirectly, regulates the expression of a broad range of genes and signalling pathways. These include growth factors and their receptors, various hormones, cellular enzymes and effectors, matrix proteins and proteolytic enzymes, oncogenes and transcription factors such as homeobox genes (Gudas et al. 1994). We now show that retinoic acid treatment of a breast cancer cell line of the intermediate phenotype results in a dramatic epithelial differentiation characterized by a large

1 increase in cell-cell adhesion strength, increased expression of β -catenin protein and its movement to a
2 triton insoluble pool at cell-cell contact sites.

3

4 **Materials and Methods**

5 **Antibodies, retinoids and other reagents**

6 Polyclonal antibody to β -catenin was a gift of Barry Gumbiner and has been described previously

7 (McCrea et al. 1993). Monoclonal antibody to β -catenin was obtained from Transduction Laboratories

8 (Lexington, KY). 9-cis RA, the RAR α -specific ligand TTAB and the RXR-specific ligand (SRI-1217)

9 were gifts of Marcia Dawson (SRI International) and have been described previously (Lotan et al. 1995;

10 Lehmann et al. 1992). All trans RA and the vitamin D3 analogue Ro5531 were gifts of Hoffman La

11 Roche. Staurosporine and okadaic acid were obtained from UBI (Lake Placid, New York),

12 cycloheximide, actinomycin D, calpain inhibitor and heparin were obtained from Sigma (St. Louis, Mo),

13 CMFDA was obtained from Molecular Probes (Eugene Oregon). Bisindoylmaleimide was obtained from

14 Boehringer-Mannheim Transforming growth factor β 1 and TGF β latency associated peptide (LAP) were

15 gifts of R & D Systems, and Matrigel was a gift from Dr. Hynda Kleinman. Suramin was kindly provided

16 by Dr. Anton Wellstein (Lombardi Cancer Center, Georgetown University) and conditioned medium from

17 MCF-7 cells over-expressing the erbB-2 ligand was a gift from Dr. Ruth Lupu (Lombardi Cancer Center,

18 Georgetown University).

19

20 **Cells and cell culture**

21 SKBR3 cells were obtained from ATCC and maintained in DMEM containing 10% fetal calf serum

22 (FCS) as described previously (Sommers et al. 1991). For Matrigel morphology experiments cells were

trypsinized and 100,000 cells were plated into wells of a 24 well plate each well containing 200 μ l of Matrigel which had been allowed to gel for 60 min. Generally the cells were plated in DMEM with 10% FCS containing various concentrations of retinoids or the inhibitors. In order to address whether the RA effects required serum some experiments were carried out in the absence of FCS. Cell cultures were monitored for up to 4 days. In the co-culture experiments SKBR3 cells were treated for 12 h with 10^{-7} M 9-cis RA, washed and trypsinized. Another flask of untreated cells was trypsinized at the same time. The RA-treated cells (mostly small aggregates) were labeled with the membrane impermeant dye CFDMA for 15 min as described previously, washed and equal numbers of treated and untreated cells plated in 12 well plates containing glass coverslips (Sommers et al. 1994b). Co-cultures were monitored for 48h for any morphological change in the unlabelled cells adjacent to the RA-treated fluorescent cells (see figure 2B).

Laminar flow assays for the measurement of cell-cell adhesion strength

SKBR3 cells which had been exposed to RA for 24 h or control cells were trypsinized in the presence of 5 mM Ca⁺⁺ resuspended to a density of 10^6 cells/ml in a volume of 5 ml and allowed to reaggregate for 4 h at 37°C with shaking at 150 rpm in a T75 flask. Cells were pelleted and adhesion strength measured using a laminar flow assay as described previously (Byers et al. 1995). Briefly, this involves allowing the cell aggregates to attach to glass coverslips coated with laminin and exposing them to fluid shear stress in a laminar flow chamber. The number of cells which detached from the parent aggregate at a given shear stress was measured and the adhesion strength calculated.

1 **Immunoprecipitation, Western blotting, Northern Analysis and Immunocytochemistry**

2 Sub-confluent cultures in 150 mm dishes were washed twice in isotonic buffer and lysed for 15 minutes
3 on ice in 1% Nonidet P-40 lysis buffer with 1 mM NaVO₄, 50 mM NaF, 20 µg/ml PMSF, 2µg/ml
4 leupeptin and 1 µg/ml pepstatin and 1 µg/ml aprotinin (Lechleider et al. 1993). After clarification for 15
5 minutes at top speed in a microfuge at 4°C, lysates were pre-cleared with 100 µl protein A Sepharose
6 (Pharmacia) and 20 µl normal rabbit serum twice for one hour. Protein content was measured by a
7 colorimetric method (Bio-Rad). Equal amounts of protein (approximately 1 mg) were incubated with 2 µl
8 of anti-β-catenin rabbit antiserum or non-immune serum. Immune complexes were precipitated with 10
9 µl protein A sepharose, washed 6 times in lysis buffer and resuspended in loading buffer. Western
10 blotting was carried out as described previously using either the polyclonal β-catenin antibody at 1:5000
11 or the monoclonal antibody (Transduction Laboratories). On some occasions cells were extracted
12 directly in reducing sample buffer as described previously with the exception that the samples were boiled
13 for 30 min prior to SDS gel electrophoresis (Sommers et al. 1994b). The extended boiling (increased
14 from 5 to 30 min) in the presence of reducing agent significantly reduced the viscosity of the samples
15 making treatment with DNAase or sonication unnecessary.

16 Immunocytochemistry was carried out with or without Triton extraction as described previously
17 (Sommers et al. 1994b). Total RNA was isolated from SKBR3 cells at various times following treatment
18 with RA as described previously (Sommers et al. 1991). Blots were probed with a random primer-
19 labelled Eco-R1 fragment from the coding region of human β-catenin.

Results

Retinoic acid promotes SKBR3 cell epithelial differentiation and increases cell-cell adhesion strength

Morphology on plastic. We showed previously that untreated SKBR3 cells growing on plastic express very low levels of E-cadherin and β -catenin protein and exhibit a rounded morphology (Sommers et al. 1991; Sommers et al. 1994b). Following treatment with 9-cis RA, all trans RA or an RAR-specific ligand the cells exhibited a marked change in appearance which was characterized by an increase in cell-cell and cell-substratum contact (Figure 1). Following 2-4 days of RA treatment SKBR3 cells were also growth inhibited (Table 1). SKBR3 cells were also growth-inhibited by the vitamin D₃ analogue 5531 (Table 1). However, this agent did not result in the pronounced morphological change observed following RA treatment (Table 2). SKBR3 cells are known to over-express the tyrosine kinase receptor erbB-2 and to respond to high levels of the erbB-2 ligand with decreased proliferation and an increase in the expression of breast-specific differentiation markers (Bacus et al. 1992; Lupu et al. 1992b; Lupu et al. 1992a; Stabler et al. 1994). Treatment of SKBR-3 cells with conditioned medium containing erbB-2 ligand did result in a morphological change as has been described by others (Bacus et al. 1992). Cells were flatter, indicating an increase in cell-substratum contact and extended many processes. However, there was no evidence of increased cell-cell contact (Table 2).

Matrigel morphology and laminar flow assays. In previous studies we showed that SKBR3 cells exhibited a rounded appearance when growing in the basement membrane gel Matrigel and did not form

1 fused cell colonies as was the case with other "more differentiated" lines such as MCF-7. Following 12 h
2 of RA-treatment SKBR3 cells formed fused cell colonies indistinguishable from those formed by MCF-7
3 or T47D cells (Table 2 Figure 2A). No effect on Matrigel morphology was observed if the cells were
4 treated with Ro5531, TGF β or erbB-2 ligand indicating a specificity for RA (Table 2). The fused
5 morphology in Matrigel is generally assumed to represent an increase in cell-cell adhesion strength
6 although no studies have actually addressed this. We recently developed methodology with which to
7 investigate the strength of cell-cell adhesion directly (Byers et al. 1995). Using this laminar flow assay
8 we found that RA treatment of SKBR3 cells resulted in a dramatic increase in the strength of cell-cell
9 adhesion from less than 7 dyn/cm² to 100 dyn/cm².

0

1 **The effects of retinoic acid on SKBR3 cell epithelial differentiation require RNA and protein**
2 **synthesis but not serum.**

3

4 Not surprisingly, treatment of SKBR3 cells with actinomycin D or cycloheximide prevented the effects
5 of RA on Matrigel morphology indicating a requirement for RNA and protein synthesis (Table 2).
6 Removal of serum from the medium followed by 5 washes in serum-free medium did not affect the
7 Matrigel morphology following RA treatment indicating that factors present in serum are not necessary
8 for RA to exert its effect on SKBR3 phenotype (Table 2).

The effects of retinoic acid on SKBR3 cell epithelial differentiation are blocked by low calcium medium and the serine kinase inhibitors staurosporine and bisindoylmaleimide

The effects of RA on Matrigel morphology could be reversed by growing the cells in medium containing 50 μM Ca^{++} indicating a mechanism involving Ca^{++} -dependent adhesion (Table 2, Figure 2A). The broad spectrum kinase inhibitor staurosporine (10 nM for 16h) also reversed the effects of RA on Matrigel morphology whereas the serine phosphatase inhibitor okadaic acid was without effect when used alone (Table 2). Staurosporine is a rather broad spectrum kinase inhibitor which can inhibit serine kinases such as protein kinase C (PKC), cAMP and cGMP-dependent kinases, as well as certain receptor and non-receptor tyrosine kinases such as *src* and EGF-R at the doses used in this study. In order to further define the role of kinase activity in mediating the effects of RA we tested the effects of the tyrosine kinase inhibitors herbimycin and genistein and the specific PKC inhibitor bisindoylmaleimide (Toullec et al. 1991). Neither herbimycin nor genistein affected the Matrigel morphology of SKBR3 cells (Table 2). Bisindoylmaleimide (10 μM) completely reversed the RA-induced morphological changes (Table 2, Fig. 2A). This agent is quite specific for PKC, the inhibition constants for other known serine/threonine kinases and tyrosine kinases are at concentrations 2-6 orders of magnitude higher than those required to block PKC (Toullec et al. 1991). These results point to a role for serine kinase activity, possibly PKC, in mediating some aspect of the RA-induced morphological changes. However, it should be pointed out that bisindoyl maleimide may inhibit the activity of an unknown kinase.

1 **The Differentiation-Promoting Effects of 9-cis Retinoic Acid do not appear to be Transmitted**
2 **Directly to Other Cells.**

3
4 In other systems RA exerts some of its effects through the increased expression of proteins that are
5 secreted, such as TGF β (Glick et al. 1991; Wakefield et al. 1990; Sporn and Roberts, 1991). Although
6 TGF β alone is not responsible for the epithelial differentiation that was observed in our experiments
7 (Table 2) it is still possible that TGF β is required (but not sufficient) for the epithelial differentiating
8 effects of RA. To test this, cells were treated with RA in the presence of the TGF β inhibitor, LAP, at a
9 concentration sufficient to completely block TGF β action. Table 2 indicates that LAP does not reverse
10 the effects of RA on Matrigel morphology. This experiment excludes a role for TGF β in mediating the
11 effects of RA but does not rule out some other secreted factor. To test this, cells were treated with 9-cis
12 RA (10^{-7} M) for 4 hours the minimum time required for the morphological changes to be observed (after
13 16 h). Cells were washed (5X) and a small volume (5ml for a T75 flask) fresh medium, without RA was
14 added. 24h later conditioned medium was collected, centrifuged to remove cell debris and added to fresh
15 cells. The cells that had been treated with RA for only 4 h underwent the characteristic morphological
16 change after 16 h. However, cells which were treated with conditioned medium collected from these cells
17 did not exhibit epithelial differentiation (Table 2). Since it is possible that the effects of RA may be
18 mediated by a cell surface molecule or one with limited local diffusion, perhaps something acting via the
19 extracellular matrix, we performed co-culture experiments similar to those carried out by Brown et al. in
20 their studies of the local paracrine effect of the *wingless* gene product (Jue et al. 1992). In order to
21 conclusively differentiate RA-treated cells from untreated cells we took advantage of the dye CMFDA
22 that we used previously in a similar situation (Sommers et al. 1994b). Once it has entered cells, CMFDA

is esterified and cannot pass through the cell membrane or gap junctions to other cells and acts as a permanent cell marker. We labeled RA treated cells with CMFDA and co-cultured them with untreated cells as described in the Materials and Methods. Following 24 h of co-culture the RA-treated cells were clearly visible as fluorescent cell aggregates in a field of non-fluorescent cells (Figure 2B). If the effects of RA were transmitted via the cell surface or via a molecule with limited diffusion potential we would have expected a marked morphological change in non-RA treated cells adjacent to the fluorescent aggregates. This was not the case. Although the fluorescent cells previously treated with RA maintained their characteristic morphology for up to 4 days there was no obvious change in the appearance of adjacent or more distant cells which had not been treated with RA (Table 2, Figure 2B).

Retinoic Acid Increases β -catenin protein levels and Movement to a Triton Insoluble Pool at Cell-cell Contact Sites but does not increase steady state mRNA levels.

We showed previously that SKBR3 cells do not express E-cadherin but that increased staining of an E-cadherin-like molecule at the adherens junction occurred upon treatment with RA (Sommers et al. 1991; Anzano et al. 1994). Pierceall et al. have now demonstrated that SKBR3 cells have a homologous deletion of the E-cadherin gene (Pierceall et al. 1995). This accounts for the lack of E-cadherin immunoreactivity observed in our earlier studies in which we used a monoclonal antibody and indicates that the molecule which was influenced by RA and detected by a polyclonal antibody is not E-cadherin (Anzano et al. 1994). Others have reported that SKBR3 cells express detectable amounts of E-cadherin RNA as detected by Northern analysis (D'Souza and Taylor Papadimitriou, 1994). Although the identity of the E-cadherin-like SKBR3 cadherin remains a mystery the demonstration that it becomes membrane-

1 associated following RA-treatment suggests a change in the expression or function of a cadherin-
2 associated molecule necessary for its localization at the cell junction. We showed previously that SKBR3
3 cells express normal levels of the cadherin-associated proteins α -catenin and plakoglobin but expressed
4 very low levels of β -catenin protein. In the present study we also show that SKBR3 cells express low
5 levels of β -catenin protein (Figs. 3 and 4). Immunostaining confirmed that most SKBR3 cells were
6 completely negative for β -catenin, however approximately 5% of the cells were strongly positive (Fig. 3).
7 In the few positive cells, β -catenin was diffusely distributed throughout the cytoplasm and was not
8 restricted to the cell membrane or cell-cell contact sites. Following RA treatment there was a marked
9 increase in the overall staining intensity of β -catenin. Most of the β -catenin was present at the cell
10 membrane at points of cell-cell contact. Although it was present at the adherens junction it was not
11 restricted there, instead, β -catenin was present throughout the basolateral membrane of the cells. The
12 membrane-associated β -catenin was stable to triton extraction indicating that it was linked to the actin
13 cytoskeleton (Fig. 3C). Since we have not identified the SKBR3 cadherin we do not have reagents with
14 which to investigate directly cadherin/catenin interactions in SKBR3 cells and cannot determine whether
15 RA alters the association of β -catenin with the SKBR3 cadherin. The increased β -catenin protein
16 expression observed by immunocytochemistry was confirmed by western analysis (Figure 4). Maximum
17 β -catenin protein levels were observed following 48h of treatment with RA at a concentration of 10^{-7} M.
18 In contrast, RA had no effect on β -catenin steady state mRNA levels (Fig. 4C).

RA increases β -catenin protein stability

The increased β -catenin protein levels that occur after RA-treatment could be the result of increased protein synthesis or decreased degradation. In order to test this we took advantage of the ability of calpain inhibitor to reversibly prevent β -catenin protein degradation in SKBR3 cells (unpublished observations). SKBR3 cells treated with calpain inhibitor for 4 hours accumulate β -catenin protein (Figs. 5A, 5B). The level of β -catenin present following this treatment is the same in control cells and in cells treated for 48 hours with RA indicating that RA does not increase the rate of β -catenin protein synthesis. Following a chase in medium without calpain inhibitor the amount of β -catenin present in control cells decreased rapidly with a half life of less than 1 hour and no protein was detectable after 8 hours (Fig. 5A). In contrast, in cells treated with RA β -catenin half life was approximately 2 hours with protein still detectable after 24 hours of chase (Fig. 5B). These results are similar to those observed for the wingless-mediated stabilization of armadillo protein in drosophila and prompted us to investigate whether the effects of RA in our system are mediated by a member of the *wnt* family (van Leeuwen et al. 1994).

The effects of RA are not mediated by a member of the *wnt* family

Wnt-1/wingless effects are exerted very locally, perhaps via the extracellular matrix and are the result of increased armadillo protein stability (van Leeuwen et al. 1994). Although the experiments described earlier do not point to a role for such local interactions in mediating the effects of RA on Matrigel morphology (Table 2), we further addressed the potential role of *wnt-1* in mediating the effects of RA on SKBR3 Matrigel morphology and β -catenin protein levels by testing the effects of a known inhibitor of

1 wnt-1 action, heparin (Jue et al. 1992). Table 2 shows that heparin did not affect the ability of RA to
2 mediate a morphological change. Although heparin is a potent inhibitor of wnt-1 action it is less effective
3 at inhibiting other wnt family members. However, suramin which is an effective inhibitor of other wnts
4 and many other growth factors did not influence the effects of RA. These results strongly suggest that the
5 effects of RA on increasing β -catenin protein levels and stimulating SKBR3 epithelial differentiation are
6 not mediated by a member of the wnt family.

7

8 **Low calcium medium and serine kinase inhibitors do not affect RA-increased β -catenin protein**
9 **levels but do prevent β -catenin movement to the cell membrane**

10

11 Since staurosporine, bisindolylmaleimide and low calcium medium were able to reverse the effects of RA
12 on epithelial differentiation we expected that the RA-induced increase in β -catenin protein levels would
13 be inhibited by these treatments. However, treatment with the kinase inhibitors or with low calcium
14 medium did not prevent the RA-mediated increase in β -catenin levels as demonstrated by immunoblotting
15 (Figs. 6 and 7). Immunocytochemistry revealed high cytoplasmic levels of β -catenin under these
16 conditions but little β -catenin was observed at the cell-cell contact sites (Fig. 6). These results indicate
17 that serine kinase activity and/or the function of a calcium-dependent adhesion molecule is necessary to
18 localize β -catenin efficiently at the cell membrane. As stated earlier, at this time we cannot investigate
19 directly whether serine kinase inhibitors influence cadherin/ β -catenin association in SKBR3 cells.

Discussion

In this study we demonstrate a relationship between retinoid-induced differentiation and the functional expression of components of the cadherin/catenin-based adhesion and signalling system in breast cancer cells. β -catenin is certainly required for cadherin-based cell-cell adhesion, however, the demonstration that β -catenin associates with the tumor suppressor gene APC in the absence of E-cadherin points to an additional role for β -catenin in the control of cell proliferation(Su et al. 1993; Rubinfeld et al. 1995; Hulsken et al. 1994). β -catenin is also a substrate for at least one serine kinase as well as for several receptor and non-receptor tyrosine kinases ((Hulsken et al. 1994; Matsuyoshi et al. 1992) Lechleider et al, submitted). Collectively, these data strongly indicate that RA, cadherins and catenins, serine and tyrosine kinases are intricately involved in the homeostasis of the epithelial phenotype. Consequently, it is likely that direct or indirect perturbations of any of these systems could lead to alterations in the control of cell proliferation and of the epithelial phenotype, resulting in the development or progression of cancer.

The demonstration that a ligand which specifically activates RAR α is able to duplicate the effects of ATRA and 9cRA in stimulating the adhesive phenotype and inhibiting thymidine incorporation and cell proliferation *in vitro* unequivocally demonstrates the involvement of this class of RAR. Since RAR homodimers are not thought to be biologically relevant we ascribe this activity to heterodimeric RAR α complexes with a member of the RXR class of receptors (Mangelsdorf et al. 1993). Unlike RAR, RXR homodimers can mediate ligand-dependent actions. However, a ligand which specifically activates RXR/RXR homodimers does not mediate RA-induced epithelial differentiation.

1 **What is the mechanism of RA-induction of SKBR3 cell epithelial differentiation?**

2

3 The effects of retinoids could be observed if the cells were treated for as little as 4 hours which
4 infers a switch-like mechanism of action. That is, in this case, RA may turn on a (genetic) program
5 which, once initiated, maintains the epithelial phenotype. Inhibition of RNA synthesis by actinomycin
6 (Table 1) prevented these effects indicating a role for transcriptional activation. A recent study also shows
7 an effect of RA on the adhesive phenotype of a cancer cell line (Vermeulen et al. 1995). However, the
8 mechanism of action of RA is quite different from that observed here. In that study RA induced the
9 adhesive function of endogenous E-cadherin in an adhesion defective cell line within 30 minutes. No
10 changes in the levels of β -catenin protein were observed and the effects of RA did not require RNA or
11 protein synthesis. In contrast, the results of the present study are reminiscent of the effects of RA in
12 development. In developing systems and in other situations RA acts in part through the induction of
13 expression of secreted molecules such as TGF- β , the TGF- β family member decapentaplegic, wingless
14 and hedgehog (Wakefield et al. 1990; Sporn and Roberts, 1991; Helms et al. 1994; Gelbart, 1989). The
15 possibility that the effects of RA in our experiments are mediated via an indirect action of a secreted
16 wingless-like molecule are particularly interesting since exogenous expression of wingless or its
17 mammalian homologue wnt-1 results in increased β -catenin and plakoglobin protein expression and
18 increased cell-cell adhesion (Bradley et al. 1993; Hinck et al. 1994). Breast cancer cells are also known to
19 express several members of the wnt family (Huguet et al. 1994). However, conditioned medium
20 (concentrated up to 10X) from RA-treated cells was ineffective in mediating epithelial differentiation
21 (Table 1). Treatment of SKBR3 cells with TGF- β 1 did not result in changes in β -catenin expression or
22 epithelial differentiation (Table 1). This probably rules out an effect of RA-induced secretion of certain

molecules such as TGF- β but does not exclude a role for wnt-related proteins which have very limited diffusion and are thought to exert their action locally. However, heparin and suramin which are known to reverse the effects of wingless and other wnt family members did not reverse the RA-induced changes (Table 1). In order to generally investigate the possibility that the RA-induced effects can be transmitted to neighboring cells we co-cultured RA-treated cells with untreated cells and looked for an effect on cells within a 1-10 cell radius. These experiments are similar in design to those used by Brown and co-workers to demonstrate that wnt-1 transfected cells could transform neighboring non-transfected cells (Jue et al. 1992). In our experiments we were unable to morphologically transform neighboring non-RA treated cells by co-culturing fluorescently tagged RA-treated cells with untreated cells (Table 1; Figure 2B).* Although we cannot exclude the possibility that RA-treated cells stop producing any putative mediators of epithelial differentiation after 4 h (the time of incubation with RA), taken together, these results raise the possibility that the effects of RA on epithelial differentiation may be autonomous and not transmitted by secreted or cell surface molecules.

The effects of RA on β -catenin expression are not mediated by an increase in the levels of β -catenin steady state RNA ruling out a role for direct regulation of the β -catenin gene by RA. The increased protein levels observed following RA treatment are the result of decreased degradation of β -catenin protein. Since the effects of RA can be reversed by actinomycin D, these results indicate that RA might positively or negatively regulate the expression of genes which directly or indirectly affect β -catenin protein degradation. There are several possible mechanisms whereby RA could increase β -catenin stability. For example, RA might increase the expression of a β -catenin binding protein in the membrane (a cadherin?) which could sequester newly synthesized β -catenin protein into a pool with a longer half life. Alternatively, RA could increase the expression of genes which directly modulate β -catenin stability

1 in the cytoplasm. It is also interesting to note that the β -catenin-associated protein APC is able to direct
2 β -catenin protein for degradation (Munemitsu et al. 1995). Whether or not RA influences the expression
3 or function of a cadherin or APC remains to be determined.

4 Treatment of cells with the kinase inhibitors staurosporine and bisindoylmaleimide reversed the
5 effects of RA on epithelial differentiation and cell-cell adhesion strength. Although treatment with these
6 inhibitors does not prevent the increase in β -catenin protein levels which occurs following RA treatment it
7 does prevent the movement of β -catenin to a triton-insoluble pool at the cell membrane or results in its
8 destabilization there. Similar results were obtained following treatment of cells in low calcium-containing
9 medium to prevent calcium-dependent adhesion. These findings lead us to suggest that the effects of RA
10 on SKBR3 cell β -catenin dynamics are twofold. RA increases β -catenin protein stability and via the
11 action of a serine kinase, mediates its movement to, or stabilization at, the cell membrane. Although these
12 experiments do not directly address the nature of the kinase(s) involved, retinoids are known to stimulate
13 the activity of PKC via transcriptional and post-transcriptional mechanisms (see (Gudas et al. 1994)for
14 review). Other studies also implicate PKC activation by TPA as being a key step in E-cadherin-mediated
15 compaction of mouse embryos (Winkel et al. 1990). Both E-cadherin and β -catenin exist as serine
16 phospho-proteins and β -catenin co-precipitates with a serine kinase ((Stappert and Kemler,
17 1994)Lechleider et al submitted for publication). We also demonstrated that the specific PKC inhibitor
18 bisindoylmaleimide reversed the RA-induced morphological effects. Collectively, these results strongly
19 implicate a serine kinase, perhaps PKC, as being a key mediator of the adhesive response of SKBR3 cells
20 to RA. Although our results only address the mechanism whereby SKBR3 cell epithelial differentiation is
21 modulated by RA these cells are also growth inhibited by RA. Whether growth inhibition by RA involves
22 the β -catenin/cadherin system remains to be determined. Other agents which inhibit SKBR3 cell

proliferation such as vitamin D analogues and erb-B2 ligand do not influence β -catenin protein levels demonstrating the specificity of RA effects and indicating that, in these cases, cell growth and changes in β -catenin expression are not necessarily linked. Nevertheless the known interaction of β -catenin with the tumor suppressor gene APC implies the existance of a retinoid-modulated regulatory network which might integrate contact (adhesion)-dependent signals with those involved in the control of cell proliferation.

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1 Table 1. Effects of retinoids, the vitamin D₃ analogue Ro5531 and TGF β 1 on H-thymidine uptake
2 by SKBR3 cells

3

Treatment	% inhibition of thymidine uptake after 48h ¹
9-cis retinoic acid (10^{-7} M)	95 (0.3) ²
All trans retinoic acid (10^{-7} M)	98 (0.04)
RAR α -specific ligand (10^{-7} M)	77 (1.9)
RXR-specific ligand (10^{-7} M)	16 (0.9)
Vitamin D ₃ analogue (5531) (10^{-7} M)	90 (0.3)
TGF β 1 (5ng/ml)	15

4

5 ¹-Results are expressed as percentage reduction from non-treated control. Standard deviation of triplicate
6 values is represented in parentheses.

7 ²-Cell counts after 6 days were reduced by 75%.

Table 2. Summary of the effect of retinoids and other agents on SKBR3 morphology in Matrigel, β -catenin protein levels and localization in a Triton insoluble pool at the cell membrane

Treatment	Matrigel Morphology	β -cat protein levels	Triton insoluble β -cat
None	Separated	Low	Low
9cRA, ATRA (10^{-8} - 10^{-6} M)	Fused	High	High
R \wedge R α ligand (10^{-8} - 10^{-6} M)	Fused	High	High
RXR ligand (10^{-8} - 10^{-5} M)	Separated	Low	Low
9cRA+actinomycin D	Separated	nd	nd
9cRA+cycloheximide	Separated	nd	nd
9cRA+staurosporine (10 nM)	Separated	High	low
9cRA+bisindolylmal. (10 μ M)	Separated	High	low
9cRA+low calcium (50 μ M)	Separated	High	low
9cRA+okadaic acid (20 nM)	Fused	High	High
9cRA+no serum	Fused	High	High
9cRA+heparin (20-200 μ g/ml)	Fused	nd	nd
9cRA+suramin (50 μ M)	Fused	nd	nd
9cRA+genistein	Fused	nd	nd
9cRA+herbimycin	Fused	nd	nd
Cond. Medium	Separated	nd	nd
Cell-cell contact	Separated	Low	Low
erbB-2 ligand	Separated	Low	Low
TGF β (5ng/ml)	Separated	nd	nd
TGF β LAP (125 μ g/ml)	Separated	nd	nd
Okadaic acid (20 nM)	Separated	Low	Low

¹Cells were treated with RA for 24 hours, washed 5X and medium collected during the following 24 h added to untreated cells. ²RA-treated cells were labeled with the dye CMFDA, trypsinized and mixed with non-RA treated cells. CMFDA cannot pass between cells. Only fluorescent cells formed fused aggregates. Non-fluorescent cells adjacent to fluorescent aggregates did not exhibit any changes in morphology or β -catenin expression and localization. nd-not determined

1 **Figure 1. 9-cis Retinoic Acid Promotes Epithelial Differentiation.** Breast cancer cells (SKBR3) were
2 treated for 24h with 10^{-7} M 9-cis retinoic acid. Control SKBR3 cells exhibit a rounded morphology (A).
3 Following treatment with 9-cis retinoic acid cells exhibited a marked change to a flattened epithelial
4 morphology (B).

5

6 **Figure 2A. The Effects of RA and Other Agents on the Morphology of SKBR3 Cells Growing in**
7 **Matrigel.** Non-treated SKBR3 cells grow as aggregates of non-fused cells on the basement membrane
8 gel Matrigel (upper panel). Following 24 h of treatment with some retinoids the aggregates consist of
9 fused cells (middle panel). Some manipulations (e.g. staurosporine, low calcium) can prevent this
10 phenotypic change (lower panel) whereas others (e.g. genistein, suramin) cannot (middle panel).

11

12 **Figure 2B. The Differentiation-Promoting Effects of 9-cis Retinoic Acid Cannot be Transmitted**
13 **Directly to Other Cells.** RA-treated cells were labeled with the dye CMFDA, trypsinized and mixed with
14 non-RA treated cells. CMFDA cannot pass between cells. Only fluorescent cells formed fused
15 aggregates. Non-fluorescent cells adjacent to fluorescent aggregates did not exhibit any changes in
16 morphology or β -catenin expression and localization.

17

18 **Figure 3. 9-cis Retinoic Acid Increases Cellular β -catenin Staining and Movement to a Triton**
19 **Insoluble Pool at Cell-cell Contact Sites.** (A) Control SKBR3 cells express very low levels of β -
20 catenin. Most cells are completely negative but approximately 5% of the cells are brightly positive. In
21 the positive cells β -catenin is diffusely distributed and not present at cell-cell contact sites. (B) Treatment

with RA results in a dramatic increase in β -catenin staining of all cells. β -catenin in RA-treated cells is largely restricted to areas of cell-cell contact (B) and is insoluble in triton (C).

Figure 4. 9-cis Retinoic Acid Increases β -catenin Protein Levels in a Dose and Time-Dependent Manner but has no effect on β -catenin steady state RNA. (A) Cellular β -catenin levels were measured by Western blot analysis of SKBR3 cell detergent lysates after treatment with 10^{-10} - 10^{-6} M RA for 24 h. Maximal stimulation was observed following treatment with 10^{-7} M RA. Numbers above the indicated β -catenin band indicate fold increase from control as measured by densitometry. (B) Time course of the effects of 10^{-7} M 9-cis RA on β -catenin protein levels. Maximum stimulation was observed 24 h after treatment and levels remained elevated for at least 4 days. (C) Time course of the effects of 10^{-7} M 9-cis RA on β -catenin mRNA levels. No significant changes in β -catenin mRNA were observed following treatment with RA.

Figure 5. RA increases β -catenin protein stability. Control cells (A) or cells treated with RA for 24 h (B) were exposed to calpain inhibitor ($10 \mu\text{M}$) for 4 hours then chased in medium without calpain inhibitor for the indicated times. NP40 cell lysates were probed for β -catenin. The arrowhead indicates the β -catenin band, the large lower molecular weight band present in all lanes is a non-specific band derived from the serum present in the chase medium.

Figure 6. Staurosporine and Low Calcium Medium Prevent the Movement of RA-induced β -catenin to Cell-Cell Contact Sites. (A) β -catenin levels in control cell are very low. Following treatment with RA-staurosporine (B) or RA in low calcium medium (C), β -catenin staining is still

1 elevated but β -catenin is not present at cell-cell contact sites. Inset in (C) represents a Western analysis of
2 β -catenin levels in detergent lysates of cells growing in normal and low calcium containing medium.
3 Lane 1-standards; lane 2-control without RA; lane 3+RA; lane 4-low calcium control; lane 5-low
4 calcium+RA.

5

6 **Figure 7. The protein kinase C inhibitor bisindoylmaleimide and the broad spectrum kinase**
7 **inhibitor staurosporine do not reverse the effects of RA on β -catenin protein levels. (Left panel)**
8 Bisindoylmaleimide a specific PKC inhibitor does not prevent the RA-induced increase in β -catenin
9 protein levels. At 10 μ M, bisindoylmaleimide augments the effects of 10^{-7} M RA. (Right panel)
10 Staurosporine does not prevent the effect of RA on β -catenin protein levels.

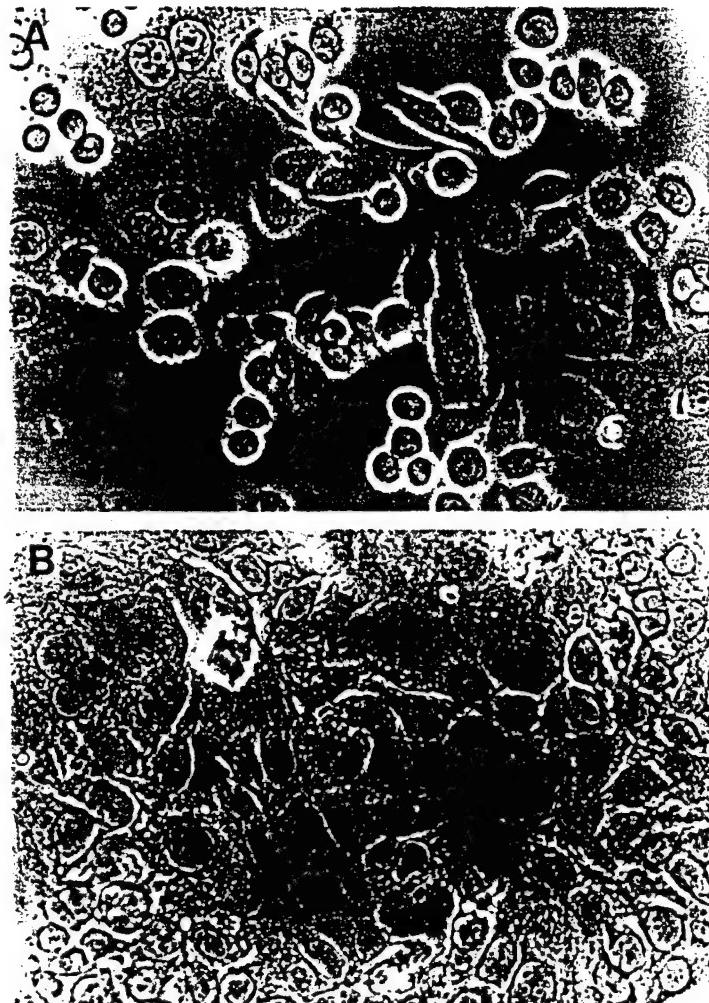


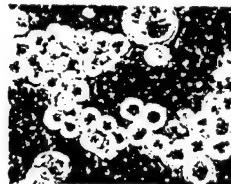
Figure 1

A

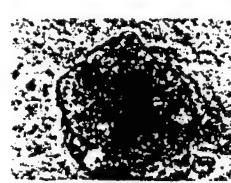
Treatment

Matrigel Morphology

Control
RXR-specific ligand
TGF β
Cond. medium
Okadaic acid
Vitamin D



9-cis RA, ATRA
RAR α -specific ligand
RA+tyr. kinase inhibitors
RA+suramin/heparin
RA-serum



RA+cycloheximide
RA+actinomycin D
RA+serine kinase inhibitors



B

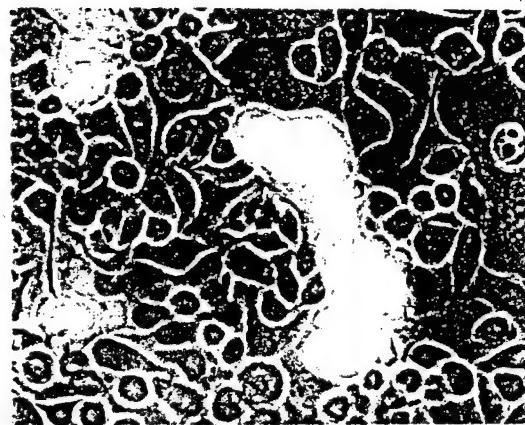


Figure 2

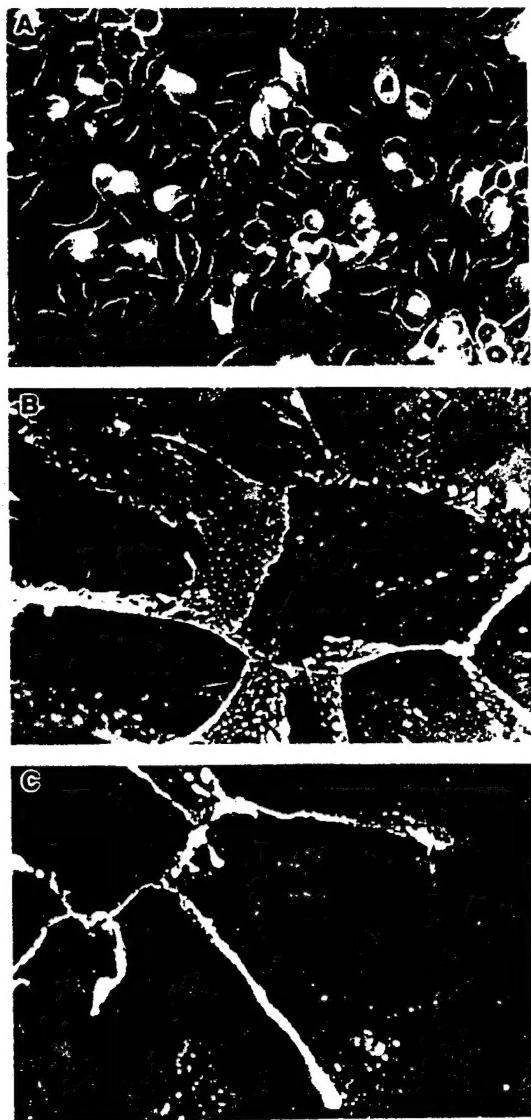


Figure 3

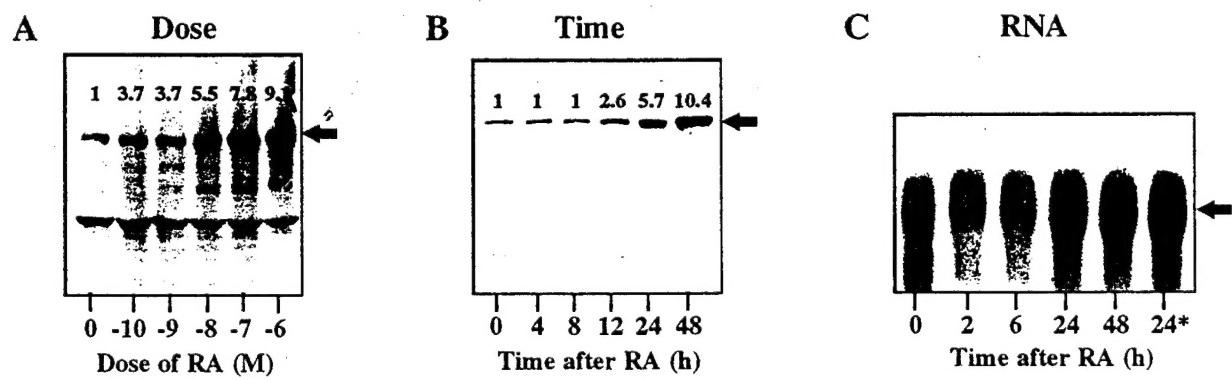


Figure 4

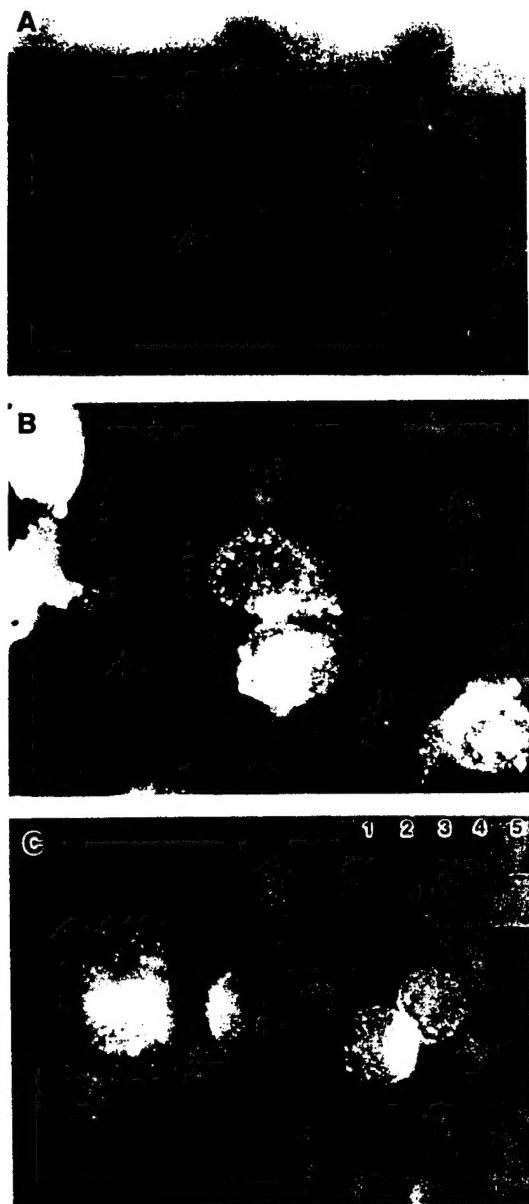


Figure 5

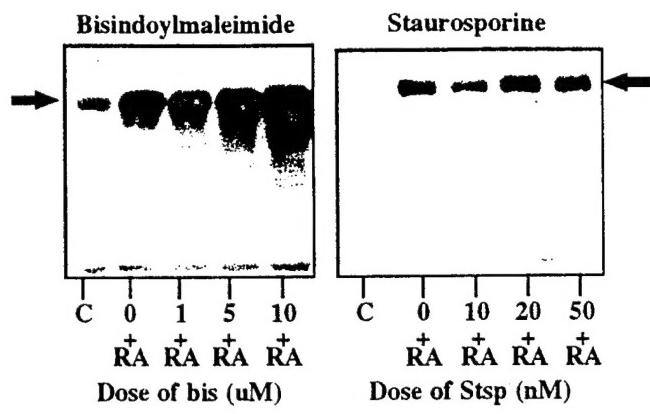


Figure 6